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Knockout of the caspase 8-associated protein 2 gene improves recombinant protein expression in HEK293 cells through up-regulation of the cyclin-dependent kinase inhibitor 2A gene

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Abstract

Cell lines used in bioproduction are routinely engineered to improve their production efficiency. Numerous strategies, such as random mutagenesis, RNA interference screens, and transcriptome analyses have been employed to identify effective engineering targets. A genome-wide small interfering RNA screen previously identified the *CASP8AP2* gene as a potential engineering target for improved expression of recombinant protein in the HEK293 cell line. Here, we validate the *CASP8AP2* gene as an engineering target in HEK293 cells by knocking it out using CRISPR/Cas9 genome editing and assessing the effect of its knockout on recombinant protein expression, cell growth, cell viability, and overall gene expression. HEK293 cells lacking *CASP8AP2* showed a seven-fold increase in specific expression of recombinant luciferase and a 2.5-fold increase in specific expression of recombinant SEAP, without significantly affecting cell growth and viability. Transcriptome analysis revealed that the deregulation of the cell cycle, specifically the upregulation of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene, contributed to the improvement in recombinant protein expression in *CASP8AP2* deficient cells. The results validate the *CASP8AP2* gene is a viable engineering target for improved recombinant protein expression in the HEK293 cell line.

Keywords

caspase 8-associated protein 2; CRISPR/Cas9; cyclin-dependent kinase inhibitor 2A; recombinant protein; RNA-seq analysis

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AUTHOR CONTRIBUTIONS

Laura Abaandou and Joseph Shiloach conceived the study and designed the experiments. Laura Abaandou performed the experiments. Ashish K. Sharma performed the bioinformatics analysis. Laura Abaandou and Joseph Shiloach wrote the manuscript. All authors collaborated on manuscript constructions and revisions.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

SUPPORTING INFORMATION

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1 | INTRODUCTION

Improved expression of recombinant proteins from mammalian cells is the desired goal being pursued through many different strategies. The main focus has been on optimizing growth and culture parameters (De Jesus & Wurm, 2011) and on modifying the properties of the producing cells (Fischer et al., 2015). The gene targets for cell modification are usually selected based on their known biological functions and the desired phenotypic outcome. For example, proapoptotic and antiapoptotic genes have been manipulated to extend the longevity of cultured cells (Baek et al., 2017; Cost et al., 2010); and genes involved in sugar transport have been manipulated to improve the metabolic efficiency of the producing cells (Inoue et al., 2010; Paredes et al., 1999). However, this approach is limited to genes with known function, whose altered expression may achieve the desired phenotype. In addition, genetic redundancy or other system-level robustness can nullify attempted modifications (Ekholm & Reed, 2000). As a result, novel approaches for identifying engineering targets are needed.

In our ongoing research, we employ noncoding RNA, microRNA, and small interfering RNA (siRNA) to identify genes whose altered expression can affect cell growth and recombinant protein expression (Inwood, Betenbaugh et al., 2018; Inwood, Buehler et al., 2018; Xiao et al., 2015; Xiao et al., 2016). Genome-wide RNA interference screens can identify targets which were not considered as engineering candidates before (Druz et al., 2013; Klanert et al., 2019; Xiao et al., 2016) while avoiding the effects of genetic redundancy and other system-level controls.

In a genome-wide siRNA screen, the caspase 8-associated protein 2 (*CASP8AP2*) gene was identified as a potential engineering target for improved recombinant protein expression. The knockdown of its messenger RNA (mRNA) by siRNA led to improved recombinant protein expression without significantly affecting cell growth and viability (Xiao et al., 2016). *CASP8AP2* encodes a protein known as “FLICE-associated large protein” (FLASH), which is involved in numerous cellular processes. In Fas-mediated apoptosis, FLASH is part of the death-inducing signaling complex (DISC) assembled following the binding of the death receptor Fas to its ligand FasL. The DISC assembly activates procaspase 8 to form proteolytic caspase 8, which triggers the extrinsic apoptotic cascade (Imai et al., 1999). FLASH, in addition to its role in apoptosis, is also involved in replication-dependent histone mRNA maturation and transcriptional regulation of histone genes (Barcaroli et al., 2006; Hummon et al., 2012; Yang et al., 2009), in the S-phase progression of the cell cycle, and in the activation of the transcription factors cMYB and nuclear factor kappa B (Alm-Kristiansen et al., 2008; Barcaroli et al., 2006; Jun et al., 2005).

In this study, the positive effect of *CASP8AP2* inactivation on recombinant protein expression was validated by knocking out this gene in HEK293 cells expressing luciferase using the CRISPR/Cas9 genome editing. The engineered cell line was evaluated for its recombinant protein expression and its growth parameters. The effect of *CASP8AP2* knockout on global gene transcription was also investigated using comparative RNA-seq analysis.

2 | MATERIALS AND METHODS

2.1 | Cell line

HEK293 stably expressing the luciferase gene of the firefly, *Photinus pyralis* under the cytomegalovirus (CMV) promoter (CMV-Luc2-Hygro HEK293; ID #CAS140901; Promega). This cell line will be referred to in this study as the parental cell line.

2.2 | Cell culture

Cells were grown adherent in Dulbecco's modified Eagle medium (Cat #11995-040; Gibco) and supplemented with 10% fetal bovine serum (Cat #S11150H; Atlanta Biologicals), 100 units/ml of penicillin, and 100 µg/ml of streptomycin (Cat #15140-122; Gibco). The cultures were incubated in a humidity-controlled incubator at 37°C and 5% CO₂.

2.3 | CRISPR/Cas9 constructs

Two plasmids encoding paired guide RNAs (gRNAs) targeting the 10th exon of the *CASP8AP2* gene, designed by transOMIC Technologies, were used in this study. The plasmids also encode carbenicillin and zeocin resistance genes and *Zoanthus* sp. green fluorescence.

Cat #TEDH-1011889, referred to in the text as plasmid 889,
gRNA-a: AAACGTAGAGTGTCAGCTGATGTGCGGAAG;
gRNA-b: ACTGGCTGTGGTGATGGTGAACCAAGGATA.

Cat #TEDH-1011892 referred to in the text as plasmid 892,
gRNA-a: CCTCCATAAGTGAACATATCTTGGGGGAAG;
gRNA-b: CTCGACTGTGTGACCTTTCTCCAGGGGTCT.

Plasmid encoding Cas9 nuclease, transOMIC (Cat #TECC1006), referred to in the text as Cas9 plasmid.

2.4 | Transfections of parental and *CASP8AP2* mutant cell lines

Twenty-four-well plates were seeded with 200,000 cells per well. The seeded cells were transfected 24 h later with the relevant plasmid(s) as follows:

For the CRISPR/Cas9 plasmid expression: Cells were cotransfected with 500 ng per well of plasmid 892 and 500 ng per well of Cas9 plasmid or with 500 ng per well of plasmid 889 and 500 ng per well of Cas9 plasmid.

For secreted alkaline phosphatase (SEAP) expression: Cells were transfected with 500 ng per well of the pSELECT-zeo-SEAP plasmid, encoding the embryonic SEAP driven by an EF-1α/HTLV composite promoter (Cat #psetz-seap; InvivoGen).

For cyclin-dependent kinase inhibitor 2A (*CDKN2A*) expression: cells were transfected with 500 ng per well of plasmid pCMV p16 INK4A (Plasmid #10916; Addgene), a gift from Bob Weinberg (Medema et al., 1995).

Lipofectamine® 2000 transfection reagent (Cat #11668-019; Invitrogen) was used in all transfections, following the manufacturer's protocol. After 2 days, cell culture media were replaced with selection media: culture media supplemented with 1 µg/ml puromycin and 10 µg/ml blasticidin for *CASP8AP2* CRISPR/Cas9 transfected cells; zeocin 200 µg/ml for SEAP transfected cells; G418 400 µg/ml for *CDKN2A* transfected cells.

2.5 | Isolation of single cells

After the *CASP8AP2* CRISPR/Cas9 transfected cell pool was propagated, single colonies were isolated using BD FACSAria™ III (BD Biosciences) green fluorescence-based flow cytometry into 96-well plates. Single clones were propagated in selection media.

2.6 | Quantification of SEAP activity

Culture supernatant samples from SEAP transfected cells were collected on Days 3 and 5 posttransfection. Alkaline phosphatase activity in the culture supernatant was quantified using the Quanti-Blue colorimetric assay (Cat #rep-qb1; InvivoGen), cells were enumerated using CEDEX HiREs cell analyzer/counter (Cat #7766; Roche), and specific SEAP activity determined.

2.7 | Sequencing of the target *CASP8AP2* genomic locus

Total genomic DNA was isolated using the DNeasy Kit (Cat #69506; Qiagen), following the manufacturer's protocol. The genomic region flanking the CRISPR target site of the *CASP8AP2* gene was amplified using Phusion® High-Fidelity PCR Master Mix with HF Buffer (Cat #MO531S; NEB) and the following primer sets:

For 889 CRISPR-transfected cells:

forward primer—CTGCCAAATCTGGAAAAGGA;

reverse primer—CAGAATCTGAGTTACTTTTAAACTTCG;

forward primer—GTGAAGAGCCCATTTGTGGT;

reverse primer—TCATGATCTACCTGCCTCAGC;

forward primer—CTAGCACTTAGGGAGGCTGA;

reverse primer—ATCAGGTCTTTTATTCCATTTGTT

For 892 CRISPR-transfected cells:

forward primer—CAAAGACTGAAAGCAAAGTTTCG;

reverse primer—ACCACAAATGGGCTCTTCAC.

Amplicons were run on ethidium bromide agarose gel, the DNA was extracted using the QIAquick® Gel Extraction Kit (Ref #28704; QIA-GEN) and sequenced using capillary DNA sequencing. Sequences were aligned with the parental sequence using the EMBOSS Water (European Bioinformatics Institute) Pairwise Sequence Alignment program.

2.8 | Luciferase activity assay

Cell viability and luciferase activity were determined using CellTiter-Glo and One-Glo luciferase luminescent assays (Cat #G7570 and Cat #E6110, respectively; Promega). Plates were read in a SpectraMax®microplate reader (Molecular Devices), at an integration time of 250 ms.

2.9 | Quantitative polymerase chain reaction analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) was completed using the SYBR GREEN protocol. Total RNA was extracted from parental cells and *CASP8AP2* knockout cells using the RNeasy kit (Cat #74101; QIAGEN). Complementary DNA (cDNA) was synthesized from total RNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Cat #K1642; Thermo Fisher Scientific). Twenty nanograms of synthesized cDNA were amplified in a qPCR using the following primers: *CASP8AP2*: forward primer—AGAGAAGAGGATGGGAAGATTG, reverse primer—GCATGGCATC AACTTCCTTAG; Luc: forward primer—GTGGTGTGCAGCGAGAAT AG, reverse primer—CGCTCGTTGTAGATGTCGTTAG; *CDKN2A* forward primer—TACTGAGGAGCCAGCGTCTA, reverse primer—AGCACCACCAGCGTGTC. The threshold and threshold cycle (C_t) values were determined automatically by RQ Manager™ software (Applied Biosystems) using default parameters. The comparative cycle threshold (2^{-C_t}) method was used to analyze the expression levels of genes examined in this study. The abundance of each gene transcript was normalized to the expression of glyceraldehyde phosphate dehydrogenase (*GAPDH*) or β -actin (*ACTB*) and expressed in arbitrary units. The relative quantification of gene expression was performed in triplicates for each sample.

2.10 | Luciferase western blot

Parental and engineered cells in confluent six-well plates were lysed using radioimmunoprecipitation assay cell lysis buffer (Cat #89900; Thermo Fisher Scientific) supplemented with 1X protease and phosphatase inhibitor (Cat #1861281; Thermo Fisher Scientific), and total protein was quantified at A280 using Nanodrop One^c (Thermo Fisher Scientific). Cell lysate samples containing 340 μ g of total protein and 100 ng of recombinant firefly luciferase (Cat #ab100961; Abcam) were electrophoresed on a 4%–12% bis-tris gel (Cat #NP0322BOX; Thermo Fisher Scientific). The separated proteins were transferred onto a nitrocellulose membrane and luciferase was detected using mouse anti-luciferase polyclonal antibodies (Cat #PA1-179; Thermo Fisher Scientific)/horseradish peroxidase-conjugated goat anti-mouse antibodies (Cat #474-1806; KPL). Mouse anti- β -actin monoclonal antibodies (Cat #A2228; Sigma) were used to detect β -actin in identical samples. The signal was developed using SuperSignal® West Pico Chemiluminescent substrate (Cat #OD187429; Thermo Fisher Scientific) and visualized in a LAS-4000 Mini Luminescent Image Analyzer (Cat #28955810; GE Healthcare). The intensity of the Western blot bands was determined using ImageJ software (NIH) and the luciferase/actin ratio and fold increase was reported.

2.11 | Growth characterization determination

Four T-25 flasks were inoculated with 7×10^5 cells in 5 ml culture media and incubated as described above. The cells were enumerated each day for 4 days using the CEDEX HiRES counter (Cat #7766; Roche). Glucose and lactate concentrations were quantified using the YSI 2900 bio-analyzer (Yellow Springs Instrument Co.).

2.12 | Cell cycle analysis

The proportion of cells in different cell cycle phases was evaluated using flow cytometry (Beckman Coulter FC500; Beckman Coulter). Cells were cultured asynchronously or synchronized by double thymidine block as previously described (Chen & Deng, 2018) and collected at 0, 2, 6, and 29 h after release from the second thymidine block. Cells (1×10^6 cells/ml) were then briefly washed twice with phosphate-buffered saline (PBS) and resuspended in 300 μ l PBS in a microtube. Seven hundred microliters of 100%, -20°C high-grade ethanol was added and the tube inverted several times. Cells were stored at -20°C until required for flow cytometric analysis. Propidium iodide (PI) staining was carried out using the FxCycle™PI/RNase Staining Solution (Cat #F10797; Life Technologies), following the manufacturer's protocol. The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson), counting 10,000 events for each sample. Data were analyzed using FlowJo software (Tree Star).

2.13 | Fas-mediated apoptosis induction and caspase 8 activity assay

Cells in the culture at a confluency of 80%–90% were treated with anti-Fas antibodies at a concentration of 500 ng/ml (Cat #ab133619; Abcam) and incubated as described above for 4 h. The cells were then harvested by trypsinization and washed with PBS. Untreated cells were used as controls.

The FLICE/Caspase-8 Colorimetric Assay kit (Cat #K113-25; BioVision) was used to quantify caspase 8 activity in treated and untreated control cells using the manufacturer's protocol, assaying 100 ng total protein per well in a 96-well plate, and read in a SpectraMax® microplate reader.

2.14 | RNA-seq analysis

Cell culture samples from biological triplicate experiments of *CASP8AP2* mutant and parental cell lines were harvested at 80%–90% confluence and total RNA was extracted using Qiagen RNeasy Kit (Cat #74101). RNA quality was assessed using Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies). Samples with an RNA integrity number of 8 or higher were processed to generate libraries for mRNA sequencing following the Illumina® TruSeq Stranded mRNA Sample Preparation Guide. cDNA libraries of six samples were loaded onto each lane of a rapid-run flow cell on a HiSeq2500 sequencer to generate paired-end sequencing reads (Illumina) for 100×2 cycles.

The fastq files were generated from bcl files, which is the primary output of Illumina HiSeq when using the bcl2fastq module on Biowulf (NIH's high performing computation system). The fastq files were processed with PartekFlow (Partek Inc.) for bioinformatic analysis. The raw reads were preprocessed by trimming bases from the 3'-end to remove the

low-quality reads. The QA/QC of the subsequent reads were checked to ensure the quality. The reads were aligned to recent transcript model hg38 (Ensembl transcript release 92) using the STAR—2.5.3a algorithm, which mapped 203,376 transcripts and 56,852 genes. The transcriptome was quantified using the Partek E/M algorithm. The differences in the sample due to technical variations, sequencing depth, gene length, and composition were minimized by performing normalization using the transcripts per million method, adding +1, and compressing data taking log₂. The principal components of the gene expression values in each population/sample of biological triplicates were closer to each other and were significantly distant from the compared samples. Statistical tests were performed on the biological triplicate data to predict the statistically significant differentially expressed genes. Analysis of variance (ANOVA) was used to compare *CASP8AP2* deleted cells with the parental cells and to calculate the fold change (FC) values. Statistically significant genes ($p < .05$ and the FC cut-off <1.5) were further evaluated for their biological significance. Gene expression data were exported from PartekFlow and imported into ingenuity pathway analysis (IPA; QIAGEN; www.qiagen.com/ingenuity) for pathway investigation. The gene ontology, overrepresented canonical pathways, regulatory networks, and upstream regulators were analyzed using the IPA knowledgebase. Based on the change in gene expression in the parental and mutant samples, pathways were predicted and ranked based on their Z -score ($-\log [p]$).

The Fastq file and processed data file were submitted to the NCBI GEO database with GEO accession number GSE143808.

3 | RESULTS

3.1 | Creating and validating *CASP8AP2* knockout in HEK293 cells using CRISPR/Cas9 genome editing

The *CASP8AP2* gene was knocked out in HEK293 cells constitutively expressing luciferase by using CRISPR/Cas9 genome editing, and a single clone was isolated by fluorescence-activated cell sorting. Analysis of the gRNA target locus in the selected clone (892–7) showed the presence of a biallelic heterozygous mutation, with one allele having a consecutive 91 basepair insertion, while the other allele had a nonconsecutive deletion of a similar length in the tenth exon of the gene. The *CASP8AP2* gene knockout in the 892–7 clone was confirmed by qPCR analysis, which showed a 20-fold reduction in the abundance of *CASP8AP2* mRNA in the knockout cells when primers targeting exon 10 were used for amplification (Figure 1a). The functional activity of FLASH, the protein encoded by *CASP8AP2*, was tested by apoptosis assay in which the activity of caspase 8 was quantified 4 h after inducing Fas-mediated apoptosis in parental- and *CASP8AP2*-deficient cells, by using anti-Fas antibodies. As shown in Figure 1b, apoptosis induction increased caspase 8 activity by 150% in the parental cell line, while caspase 8 activity in the 892–7 clone remained unchanged, confirming the absence of functional FLASH in the 892–7 clone.

3.2 | Effect of *CASP8AP2* knockout on recombinant protein expression in the HEK293 cell line

The effect of *CASP8AP2* knockout on the constitutive expression of recombinant luciferase and the transient expression of SEAP is shown in Figure 2. Compared with the parental cell line the clone deficient in the *CASP8AP2* gene, 892–7, showed a 7.4 average fold increase in specific luciferase activity (Figure 2a), which was also associated with 1.6-fold higher abundance of luciferase mRNA (Figure 2b) and a 6.9-fold higher luciferase protein (Figure 2c). The CRISPR *CASP8AP2* knockout pool from which the 892–7 clone was isolated (892 pool), had on average a 1.3-fold increase in luciferase activity compared with the parental cell line (Figure S3).

Transient expression of recombinant SEAP from the parental cell line and the *CASP8AP2*-deficient clone, 892–7, transfected with plasmid encoding SEAP is shown in Figure 2d. Specific SEAP activity in the culture supernatant of the *CASP8AP2* knockout clone, 892–7, was 2.5-fold higher than that of the parental cell line.

3.3 | Effect of *CASP8AP2* knockout on growth, metabolism, and cell cycle progression

Growth characteristics and luciferase production from parental and *CASP8AP2* knockout clone (892–7) are shown in Figure 3a,b. The growth of the two cell lines for 4 days was comparable, with average doubling times of 24 h for the parental cell line and 26 h for the *CASP8AP2* knockout clone (Figure 3a). Glucose consumption and lactate excretion were similar, with slightly higher glucose consumption (on average 0.23 g/L more) and lower lactate excretion (on average 0.28 g/L less) in the parental cell line (results not shown). Specific luciferase production during the 4-day growth period was sixfold to ninefold higher in the knockout clone, compared with the parental cells (Figure 3b). Flow cytometric cell cycle analysis on asynchronous cells showed that compared with the parental cell line, the *CASP8AP2* knockout cell line had a 9% higher proportion of cells in the G0/G1 phase and a 6% lower proportion of cells in the G2/M phase. The proportion of cells in the S-phase was only slightly different between the two cell lines (Figure 4a–c). These results were confirmed in thymidine synchronized cells in which the 892–7 clone had 17.1%, 22.7%, 2.6%, and 11.7% more cells in the G0/G1 phase than the parental cell line at 0, 2, 6, and 29 h, respectively, after releasing from thymidine block. The percentage of cells in the G2/M phase was higher in the parental cell line at all time points (Figure 4d).

3.4 | Identifying genes associated with *CASP8AP2* knockout by RNA sequencing analysis

To investigate the downstream effects of *CASP8AP2* deletion, complete transcriptomic RNA-seq data were generated from the parental and the *CASP8AP2* knockout cells. The RNA-seq analysis is summarized in Table 1. By alignment of the reads with the recent human transcript model hg38 (Ensemble transcript release 92); with a minimum overlap of 100%, 203,376 transcripts and 56,852 genes were mapped. From these genes, 3216 significant differentially expressed genes were identified by applying ANOVA to the mapped *CASP8AP2* knockout and the parental genes, using a filter with $p < .05$ and FC cut-off of $<1.5>$. Evaluation of these genes by IPA identified several overrepresented canonical pathways. Among the top 25 differentially expressed pathways, six were found to be involved in cell cycle regulation. They are: (1) cell cycle control of chromosomal replication,

(2) HIPPO signaling, (3) cell cycle G2/M DNA damage checkpoint regulation, (4) DNA methylation and transcriptional repression signaling, (5) cell cycle G1/S checkpoint regulation, and (6) p53 signaling. Additional significantly affected pathways were the eukaryotic initiation factor 2 (EIF2) signaling pathway, protein ubiquitination pathway, regulation of eukaryotic initiation factor 4 (eIF4), p70 S6 kinase, and the mammalian target of rapamycin signaling pathway (Figure 5). The most highly differentially expressed gene family was the replication-dependent histone genes. All, except two, were upregulated in the *CASP8AP2*-deleted cell line, with 72% having a 1.5-fold or higher mRNA abundance (Figure S1).

3.5 | Identification of *CDKN2A* gene as an effector of improved recombinant protein expression

As was shown above (Figure 4), the *CASP8AP2*-deficient cell line exhibited slow cell cycle progression at the G0/G1 phase. Since slower cell cycle progression is known to improve recombinant protein expression (Sunley & Butler, 2010), the differentially expressed genes involved in cell cycle progression were evaluated for their involvement in the improved recombinant protein expression in the *CASP8AP2*-deleted cell line. RNA-seq transcriptomic analysis identified nine genes involved in cell cycle progression that were at least twofold differentially expressed between the parental and the *CASP8AP2*-deleted cell lines (Figure 6). Validation of the RNA-seq data using qPCR led to the selection of the *HDAC10* and the *CDKN2A* genes for further investigation. The *CDKN2A* mRNA was 4-fold and 2.4-fold more abundant, and the *HDAC10* mRNA was 3-fold and 4-fold more abundant in the *CASP8AP2*-deleted clone, by RNA-seq and qPCR analyses, respectively (Figure 7a,b). The effect of overexpressing these two genes on luciferase expression was tested by transfecting the parental and the *CASP8AP2*-deleted cell lines with plasmids encoding them. Overexpression of *HDAC10* showed no improvement in luciferase expression (data not shown) in either cell line. Overexpression of *CDKN2A* resulted in 2.5-fold higher luciferase activity in the transfected parental cells compared with the nontransfected parental cells and 1.5-fold higher luciferase activity in the *CASP8AP2*-deleted 892–7 cells compared with the nontransfected 892–7 cells (Figure 7c). The differential effect of the overexpression of *CDKN2A* on luciferase activity between the parental cell line and the *CASP8AP2*-deleted clone (in which the *CDKN2A* gene is upregulated), suggests that the *CDKN2A* gene is instrumental in the improved expression of luciferase in the *CASP8AP2*-deleted clone. The overexpression of the *CDKN2A* gene did not significantly affect cell growth and viability (Figure 7d).

4 | DISCUSSION

The improved luciferase expression from the *CASP8AP2* knockout HEK293 cells validates the information previously obtained from the genome-wide siRNA high-throughput screen that identified this gene as a promising engineering target for improving recombinant protein expression (Xiao et al., 2016). The expression of recombinant luciferase from the *CASP8AP2* knockout clone was approximately sevenfold higher than its expression in the parental cells. This study also identifies the *CDKN2A* gene, which was upregulated in the *CASP8AP2* knockout cell line (Figure 6c), as involved in the higher luciferase expression.

The *CASP8AP2*-deleted clone showed a consistently higher proportion of cells in the G0/G1 phase and a lower proportion of cells in the G2/M phase, suggesting a G0/G1 cell cycle arrest (Figure 4).

Growth arrest at the G0/G1 phase, where the cells demonstrate higher metabolic activity, is a known strategy for improving specific productivity in mammalian cell lines (Du et al., 2015; Hendrick et al., 2001; Ibarra et al., 2003). In this phase, the cells are larger and actively express genes responsible for ribosome biosynthesis and protein translation (Bi et al., 2004; Carvalhal et al., 2003; Dez & Tollervey, 2004). Strategies for enhancing recombinant protein production based on growth arrest include overexpression of cyclin-dependent kinase inhibitors (p21^{Cip1} or p27^{Kip1}; Bi et al., 2004; Carvalhal et al., 2003; Fussenegger et al., 1997; Meents et al., 2002), the addition of cytostatic chemical agents such as sodium butyrate or dimethyl sulfoxide (Hendrick et al., 2001; Hunt et al., 2002; Hwa Chang et al., 2002; Liu et al., 2001), and maintaining mild hypothermic culture conditions (Ahn et al., 2008; Fox et al., 2004; Nam et al., 2008). However, improved specific production using these approaches is associated with reduced cell growth and requires a careful balancing of growth with protein expression to achieve overall improvements in recombinant protein yield (Sunley & Butler, 2010). The knockout of the *CASP8AP2* presented here is likely an attractive alternative for improved recombinant protein expression mediated by growth arrest at the G0/G1 phase since it offers improvements in recombinant protein expression with minimal effect on cell growth. A possible reason for this behavior is the association of *CASP8AP2* knockout with the altered expression of two apoptosis regulators: the upregulation of the antiapoptotic gene *XIAP*, a potent inhibitor of apoptosis that inhibits caspase 3, 7, and 9; and the downregulation of *DIABLO*, its proapoptotic inhibitor (Figure S1).

The potential for utilizing *CDKN2A* as an engineering target for improved recombinant protein production is also highlighted in this study. The protein encoded by the *CDKN2A* gene, P16^{INKA}, is an inhibitor of cyclin-dependent kinases 4 and 6 that play pivotal roles in cell cycle G1 phase progression. Overexpressing *CDKN2A* in parental cells led to a 2.5-fold increase in luciferase expression while its overexpression in the *CASP8AP2* knockout cells led to an additional 1.5-fold increase, an indication that this gene is likely responsible for the overall higher expression of luciferase in *CASP8AP2* knockout cells. Previous studies that focused on overexpression of p21^{cip1} and p27^{kip1} (encoded by *CDKN1A* and *CDKN1B* genes, respectively), cyclin-dependent kinase inhibitors of cyclin E-CDK and A-CDK complexes, demonstrate two-to five-fold higher recombinant protein expression (Bi et al., 2004; Carvalhal et al., 2003; Fussenegger et al., 1997) and up to 10–30-fold, when coexpressed with the differentiation factor C/EBP α and the antiapoptotic protein Bcl-xL (Mazur et al., 1998). The overexpression of *CDKN2A* in parental cells expressing luciferase reported in this study led to a 2.5-fold increase in recombinant luciferase expression, which is within the range of the reported increases in recombinant protein expression prompted by the singular overexpression of p21^{Cip1} or p27^{Kip1}. We, therefore, suggest that *CASP8AP2* knockout causes the upregulation of cyclin-dependent kinase 2A which, in turn, leads to cell cycle arrest at the G0/G1 phase, and to the improvement of recombinant protein expression. A schematic presentation of the proposed role of *CDKN2A* is shown in Figure 8.

Overexpressing the *CDKN2A* gene in parental cells improved luciferase expression by an average of 2.5-fold, which is significantly less than the seven-fold increase driven by *CASP8AP2* knockout in the 892–7 clone. Although this difference can be the result of the additional burden of overexpression of *CDKN2A*, it is possible that the upregulation of the *CDKN2A* gene is only one of many genes whose altered expression is responsible for the observed phenotype in the 892–7 clone. Future studies concentrating on identifying these genes will provide more information on the mechanism involved.

The improvement in luciferase expression in the 892–7 clone was observed at both the transcriptional and translational levels (Figure 1a). The *EIF2* signaling pathway which was highly differentially expressed between the two cell lines might be involved in the increased translation of recombinant luciferase. Among the differentially expressed genes of this pathway were the eukaryotic initiation factor 4A (*EIF4A*), and the activating transcription factor 5 (*ATF5*) that were upregulated 3.3- and 3.5-fold, respectively, in the 892–7 clone, whilst the phosphatase 1 catalytic subunit alpha (*PPP1CA*) was downregulated 3.5-fold. The *EIF4A* gene encodes a helicase that impacts translation by unwinding double-stranded RNA enabling the binding of ribosomal 40S units. Ribosomal proteins, which are involved in protein synthesis were highly differentially expressed, with a similar number of them upregulated or downregulated in the 892–7 clone (16 vs. 15). Other members of the eukaryotic initiation factor family (*EIF2A*, *2B*, 3, 5) were only marginally up- or downregulated. Since there is no apparent pattern among the differentially expressed genes involved in protein biosynthesis, further investigation will be needed to understand their role in the improved expression of luciferase.

It was previously reported that RNAi *CASP8AP2* knockdown caused cell cycle arrest in the S-phase with a consequent reduction in cell growth and viability in different cell lines, including the HEK293 cell line, (Barcaroli et al., 2006; Hummon et al., 2012; Kiriyama et al., 2009), and in vivo lethality observed in mouse embryos deficient in the *CASP8AP2* gene (De Cola et al., 2012; Minamida et al., 2014). Cell cycle arrest at the S-phase was accompanied by suppression of the nonpolyadenylated replication-dependent histone genes. In the present study, the knockout of the *CASP8AP2* gene did not significantly affect cell cycle S phase progression and cell growth and viability. This difference could be due to the mode of inactivation of the *CASP8AP2* gene: CRISPR knockout in our study versus RNAi knockdown in the previous studies. Since cells can carry out compensatory changes when a gene is knocked out to diminish potentially lethal effects (which is not usually the case with knockdowns; Bunton-Stasyshyn et al., 2019), we speculate that the upregulation of poly-adenylated, noncanonical variants of the replication-dependent histones in the 892–7 clone (Figure S2), is a way for the cells to compensate for the suppression of canonical replication-dependent histones as a result of *CASP8AP2* knockout. Reduced synthesis of replication-dependent histones has been shown to be responsible for the S-phase cell cycle arrest and eventual cell death when *CASP8AP2* was knocked down by RNA interference (Bongiorno-Borbone et al., 2010). Another study in which the *CASP8AP2* gene was knocked out in embryonic stem cells agrees with our findings, with no significant changes in cell growth and viability observed in the *CASP8AP2* knockout cells (Minamida et al., 2014).

CASP8AP2 has been associated with multiple cellular functions. Its critical role is associated with Fas-mediated apoptosis, which was confirmed in this study by the absence of caspase 8 activation following the induction of Fas-mediated apoptosis in the *CASP8AP2* knockout clone (Figure 2b). The finding that *CASP8AP2* knockout led to the upregulation of noncanonical replication-dependent histone genes (Figure S2) supports the findings of Hummon et al. (2012), where there was a significant enrichment of histone transcripts in *CASP8AP2*-deficient colorectal cancer cells. The work presented here recommends not just a tool for improved recombinant protein expression but also suggests a possible mechanism for the improved phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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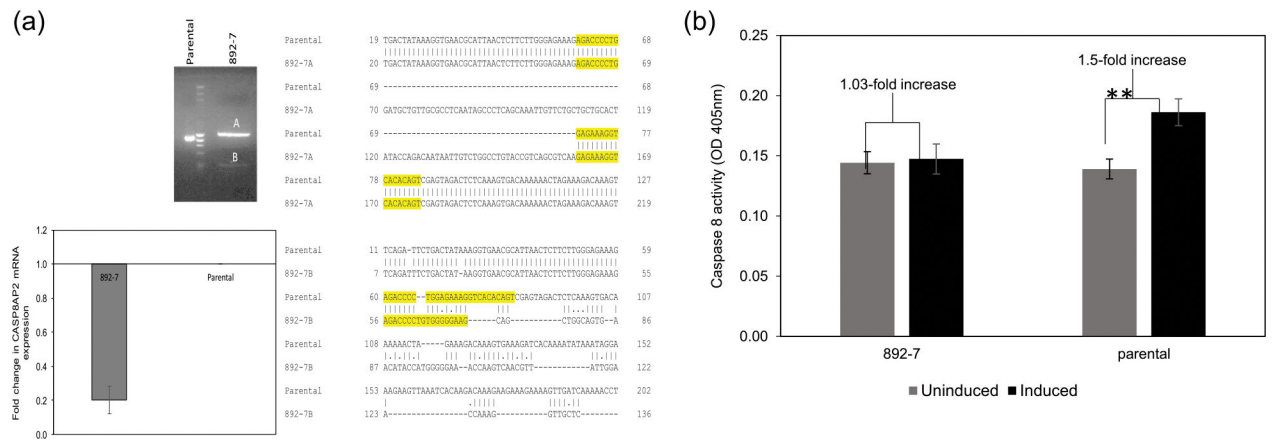
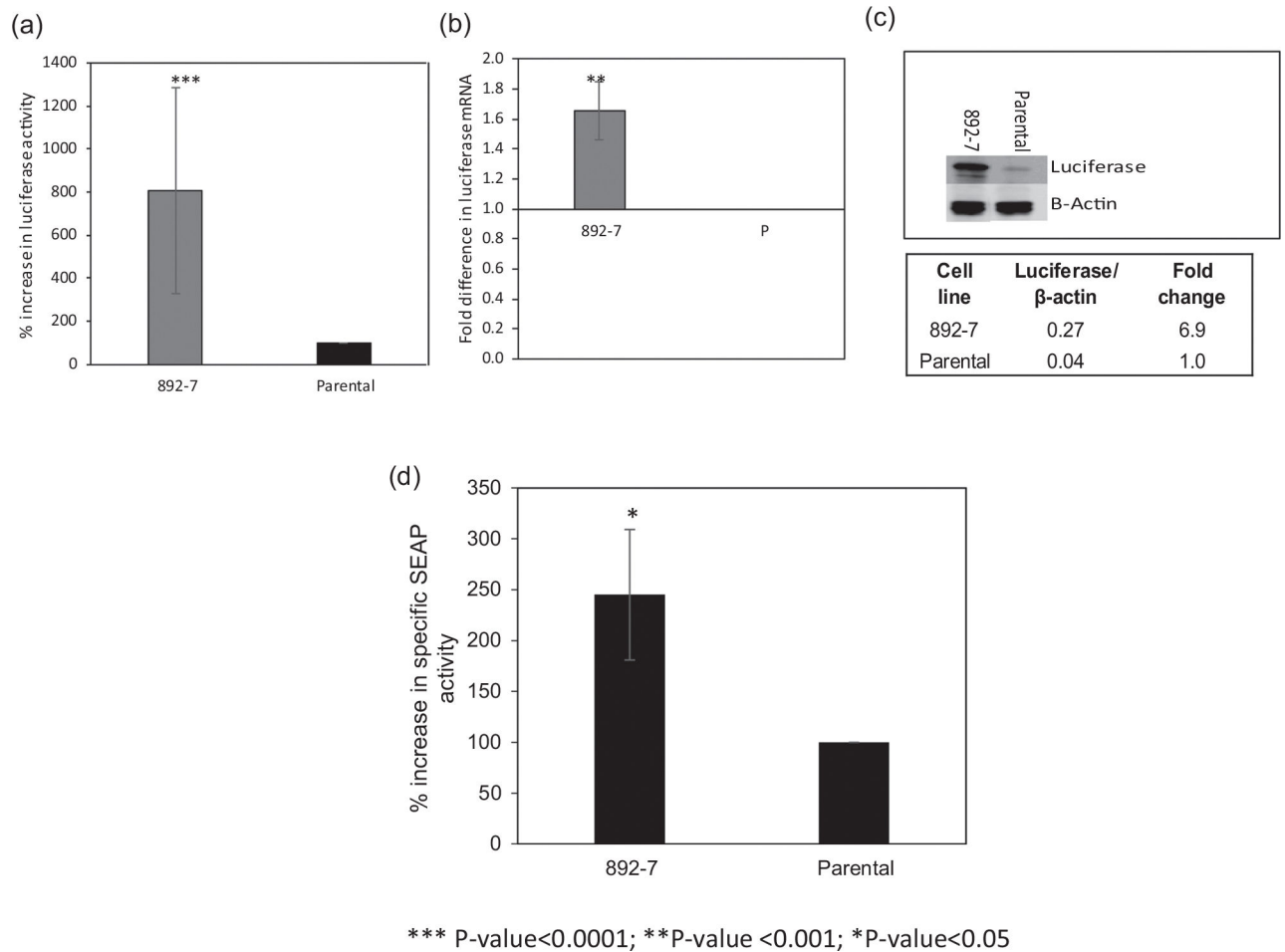
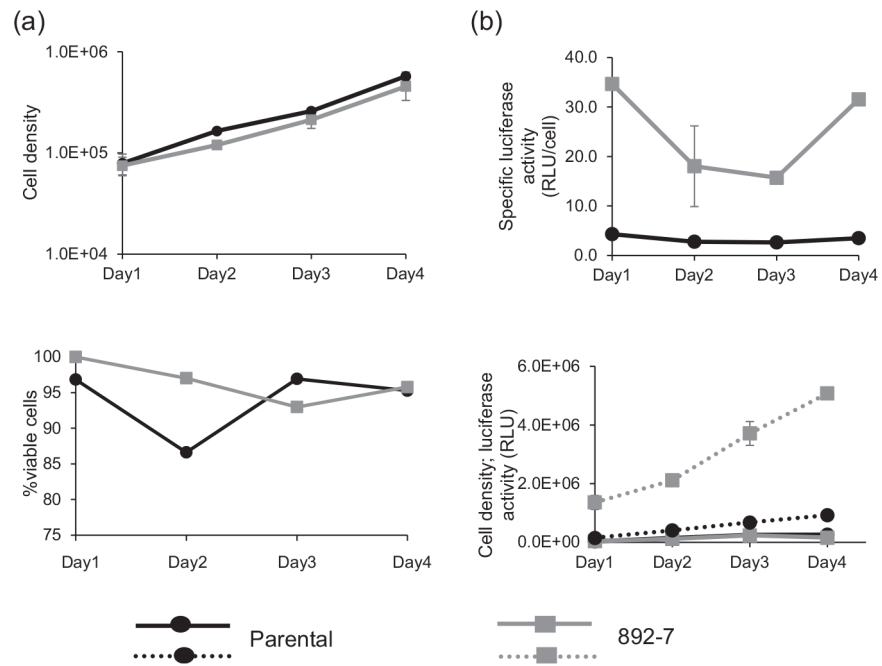


FIGURE 1. Knockout of the *CASP8AP2* gene. (a) Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified *CASP8AP2* locus in the parental and the 892-7 cell lines, and EMBOSS WATER sequence alignment of both alleles with the parental sequence. Guide RNA (gRNA) target sequence is highlighted. *GAPDH*-normalized qPCR analysis of *CASP8AP2* messenger RNA (mRNA) abundance in parental and *CASP8AP2* knockout clone, 892-7, using primers targeting the gRNA locus for amplification. (b) Caspase 8 activity in cell lysates of control (uninduced) and apoptosis-induced (induced) 892-7 and parental cells (** $p < .001$)

**FIGURE 2.**

Luciferase and secreted alkaline phosphatase (SEAP) production from the parental cell line and the *CASP8AP2* knockout clone, 892-7. (a) Luciferase enzymatic activity (***) $p < .0001$). (b) *GAPDH*-normalized luciferase messenger RNA (mRNA) abundance determined using real-time quantitative polymerase chain reaction (** $p < .001$). (c) Luciferase expression by Western blot analysis. (d) Alkaline phosphatase activity in the cell culture supernatant of cells transfected with a plasmid encoding the SEAP gene (* $p < .05$). Results represent the average of at least three biological replicates, and error bars represent the standard deviation

**FIGURE 3.**

Growth and luciferase production in the 892-7 clone and parental cell line for 4 days growth period. (a) Cell density and viability. (b) Specific and total luciferase production. Graphs are representative of three separate growth and production experiments, and error bars represent the standard deviation

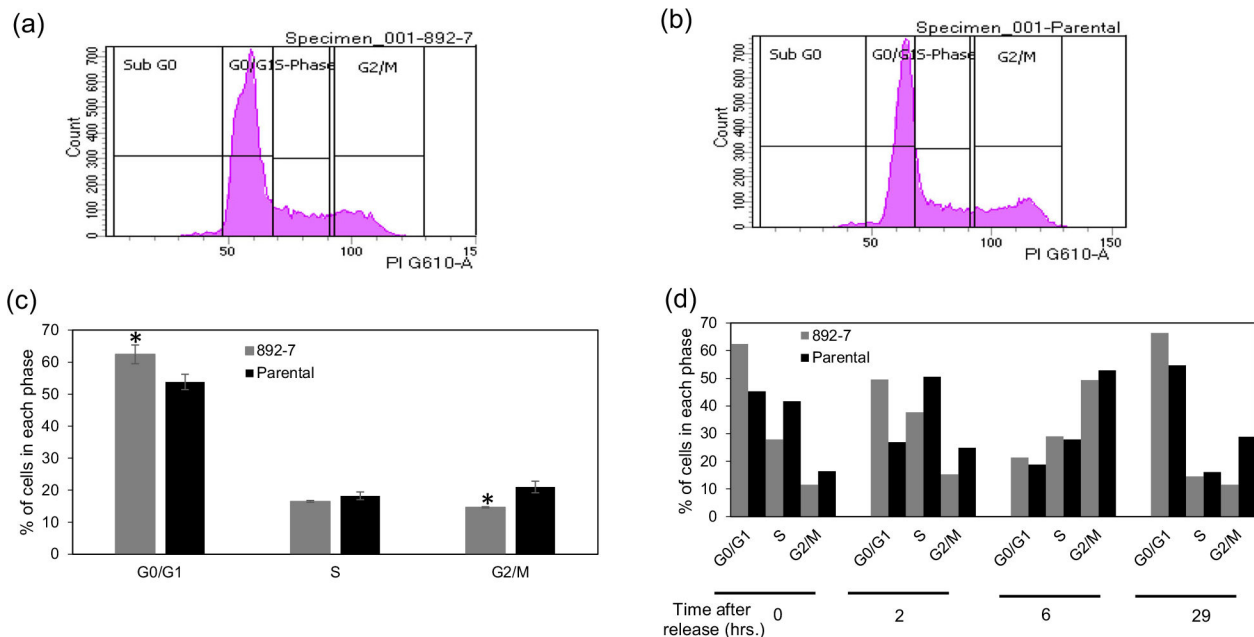


FIGURE 4. The effect of *CASP8AP2* knockout on cell cycle progression by FACS-based, propidium iodide cell cycle analysis. (a) Knockout clone. (b) Parental cells. (c) Percentages of cells in the G1, S, or G2/M in asynchronous cultures calculated using BD FACS DIVA software. Results represent the average of three analyses, and error bars represent the standard deviation (* $p < .05$). (d) Parental and 892-7 cells were treated with thymidine to synchronize the cell cycle. After release from the thymidine block, the cells were harvested at the indicated time points and cell cycle progression was analyzed using a cytometer

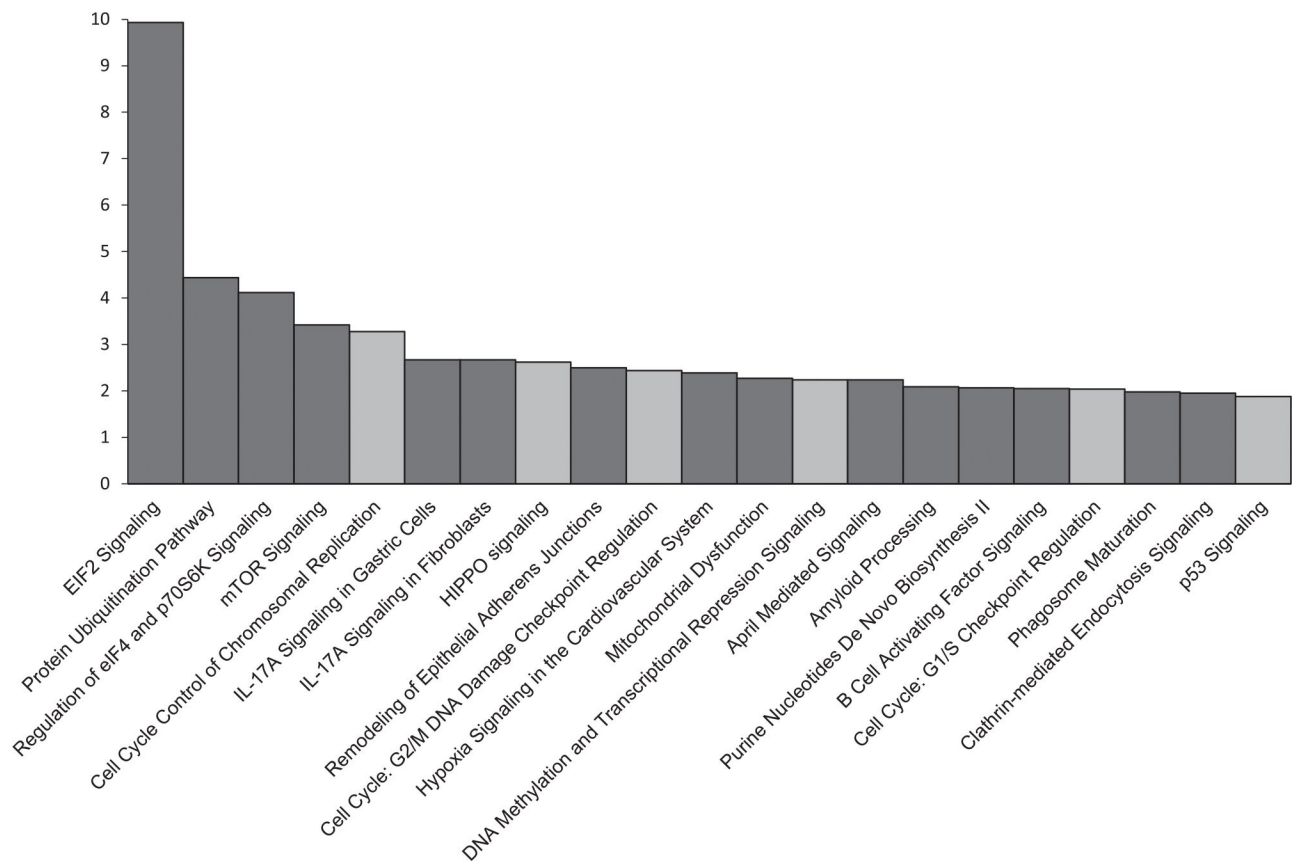


FIGURE 5.

Pathway ranking based on Z -score following enrichment analysis of the differentially expressed genes between the *CASP8AP2* knockout and the parental cell lines. Gray bars represent pathways involved in the cell cycle

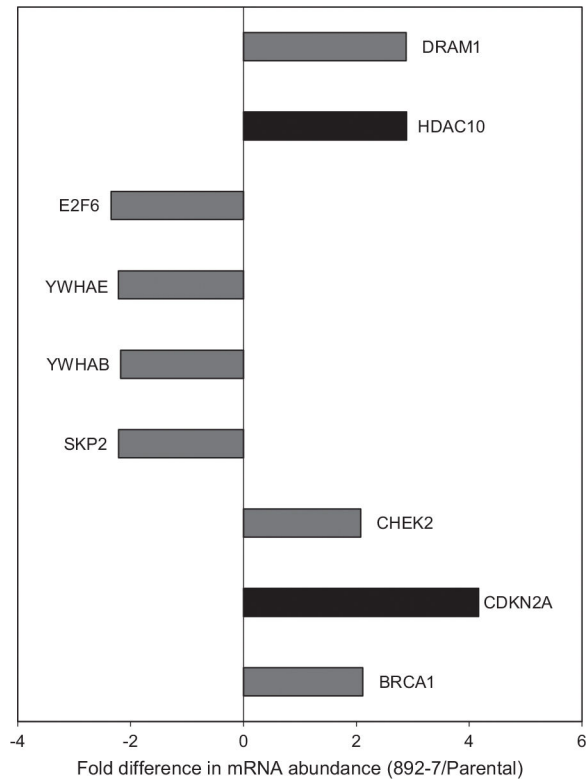
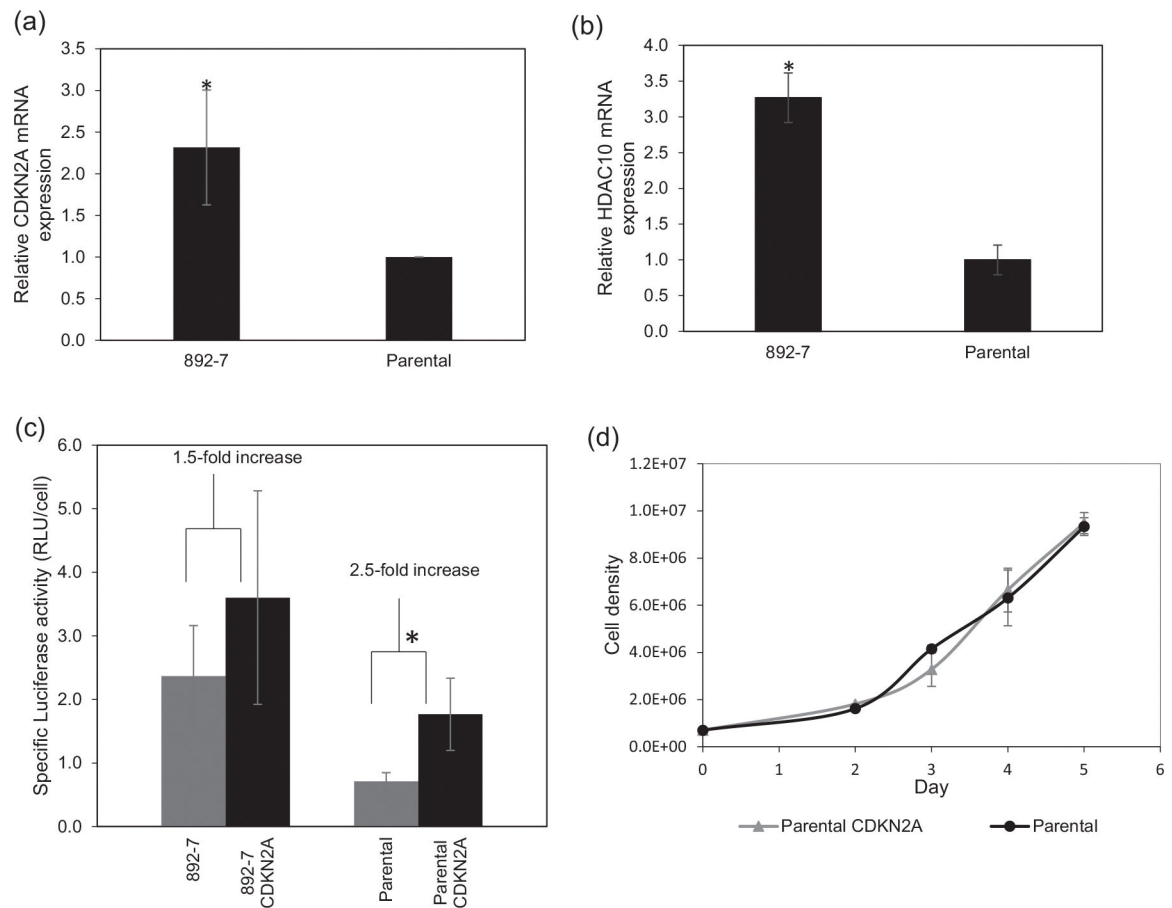


FIGURE 6. RNA-seq analysis of genes involved in the cell cycle. Differentially expressed cell cycle-related genes with more than the 1.5-fold difference in messenger RNA (mRNA) abundance between the mutant clone, 892-7, and the parental cell line with $p < .05$

**FIGURE 7.**

(a and b) Relative *CDKN2A* and *HDAC10* expression in parental and *CASP8AP2* knockout HEK293 cells determined in a *GAPDH*-normalized real-time quantitative polymerase chain reaction. (c) Effect of overexpressing *CDKN2A* on luciferase activity in parental (parental *CDKN2A*) and *CASP8AP2* knockout (892-7 *CDKN2A*) HEK293 cells (* $p < .05$). (d) Effect of *CDKN2A* overexpression on cell growth of the parental cell line. Results represent the average of at least three biological replicates, with error bars representing standard deviation

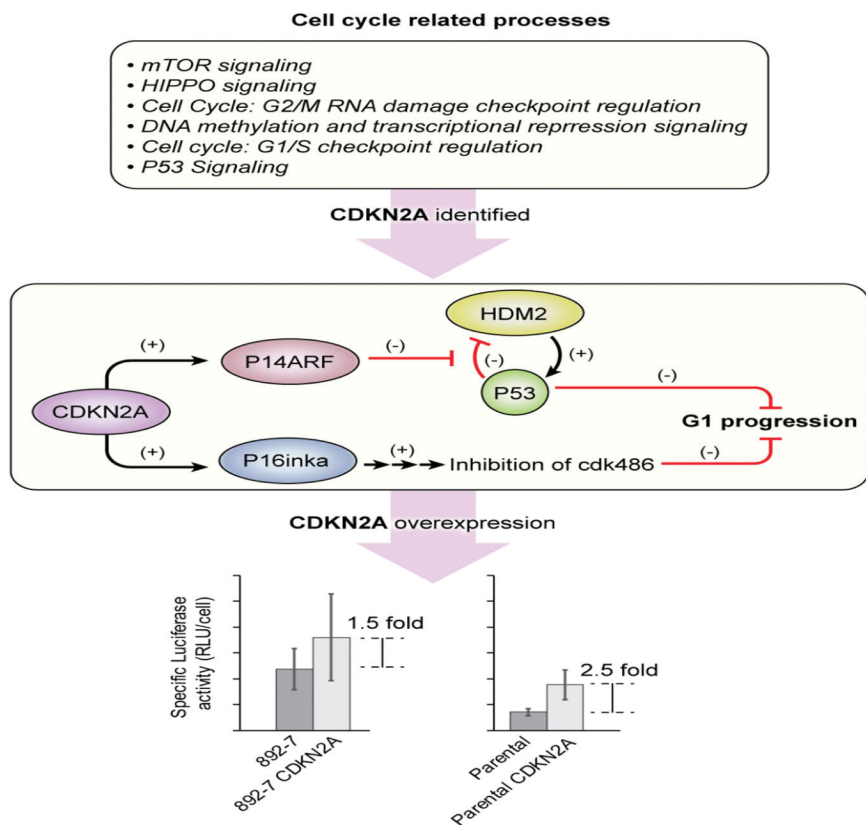


FIGURE 8. Identification of the *CDKN2A* gene as an effector for recombinant protein expression in the *CASP8AP2* deficient 892-7 clone

Summary of raw and processed RNA-seq reads generated from *CASP8AP2* mutant clone, 892-7, and parental RNA samples

TABLE 1

Sample name	Total reads (10e6)	Total alignments (10e6)	Aligned (%)	Coverage(%)	Avg. coverage depth
<i>CASP8AP2</i> mutant	17.94	40.83	95.19	5.11	25.31
<i>CASP8AP2</i> mutant	74.56	16.42	94.67	10.16	51.34
<i>CASP8AP2</i> mutant	18.95	42.17	95.31	5.51	24.28
Parental	10.53	23.9	97.98	3.46	21.87
Parental	18.85	42.85	97.56	4.60	29.60
Parental	15.78	34.73	97.68	5.26	21.03