A cellular stress response induced by the CRISPR/dCas9 activation system is not heritable through cell divisions.

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ABSTRACT

The CRISPR/Cas9 system can be modified to perform 'epigenetic editing' by utilizing the catalytically-inactive (dead) Cas9 (dCas9) to recruit regulatory proteins to specific genomic locations. In prior studies, epigenetic editing with multimers of the transactivator VP16 and guide RNAs (gRNAs) was found to cause adverse cellular responses. These side effects may confound studies inducing new cellular properties, especially if the cellular responses are maintained through cell divisions - an epigenetic regulatory property. Here we show how distinct components of this CRISPR/dCas9 activation system, particularly untargeted gRNAs, upregulate genes associated with transcriptional stress, defense response, and regulation of cell death. Our results highlight a previously undetected acute stress response to CRISPR/dCas9 components in human cells, which is transient and not maintained through cell divisions.

INTRODUCTION

The prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) system has been extensively used for eukaryotic genome editing, allowing precise point mutations, insertions and deletions, as well as epigenetic editing.[1-3] Tempering the the promise of CRISPR/Cas9 systems is the concern of off-target effects. The Cas9 nuclease protein has been shown to bind promiscuously across the genome,[4] resulting in undesirable insertion-deletion events as a consequence of this off-target cleavage.[5]

Epigenetic editing uses dCas9 (dead Cas9), a mutated Cas9 devoid of endonuclease activity, allowing the recruitment of effector proteins to specific loci without causing mutations at those sites. Over time, different CRISPR activation (epigenetic editing) systems have been proposed and compared in regards to their efficacy and off-target effects.[6] The first constructs consisted of the standard activator VP64 (four copies of VP16) linked to the C-terminus of dCas9.[7, 8] VP16 is a viral protein that forms a transcriptional regulatory complex in host cells to induce early gene transcription upon herpes simplex infection.[9] Subsequent CRISPR activation systems have been developed, many of them expressing VP16 repeats (VP64 or VP160), either fused to dCas9[7, 10-13] or recruited by protein tagging and programmable RNA scaffolds.[14, 15] Off-target activation has not been detected using CRISPR activation, suggesting that guide RNA (gRNA) sequences are not inducing off-target recruitment of dCas9 leading to gene activation. However, a prior study points to a possible side effect of epigenetic editing using VP64 that involves the downregulation of the Interleukin 32 gene (*IL32*).[7] Moreover, when produced via *in vitro* transcription, CRISPR gRNAs triggered side effects related to the innate immune response in human cells, with the upregulation of genes involved in the type I interferon response.[16, 17]

Given these potential side effects of epigenetic editing, we aimed to investigate the genome-wide, off-target effects of the CRISPR components on human transcriptional regulation. Here we examined the gene expression effects of distinct components of a VP16-based CRISPR/dCas9 activation system, by analyzing cells transiently transfected with different

71 combinations of dCas9, gRNAs and VP16 repeats, applying normalized transfected DNA
72 amounts, and selection of positively transfected cells. This strategy allowed us to characterize
73 a previously undetected acute stress response to the CRISPR/dCas9 components in human
74 cells.

75

76 **MATERIAL AND METHODS**

77 **Plasmid construction**

78 To generate the dCas9 vectors, plasmid pAC154-dual-dCas9VP160-sgExpression (Addgene
79 plasmid # 48240)[18] was linearized to introduce the 2A-GFP sequence downstream to the
80 dCas9-VP160 fusion. Reverse complement oligonucleotides were annealed and amplified to
81 generate the 2A sequence. The GFP sequence was amplified by PCR from plasmid pBI-
82 MCS-EGFP (Addgene plasmid #16542)[19] and all fragments were Gibson assembled to
83 provide the sgRNA-dCas9-VP160-2A-GFP vector. Additional steps of plasmid digestion, gel
84 purification, and Gibson assembly were then applied to the resulting vector. In this way,
85 distinct CRISPR components were sequentially removed to generate the vectors sgRNA-
86 dCas9-2A-GFP and sgRNA-2A-GFP.

87 A gRNA cloning vector (Addgene plasmid #41824) was used as the gRNA empty backbone
88 and for cloning the gRNA sequences as previously described.[20] The vector was linearized,
89 then reverse complement oligonucleotides containing the 19-nucleotide gRNA target
90 sequence and the gRNA scaffold were annealed and Gibson assembled into the vector to
91 generate individual gRNAs1-6. The gRNA sequences (**Supplementary Table 1**) were
92 selected as those with the highest scores and shortest distance to the TSS using the CRISPR
93 design tool crispr.mit.edu. Plasmid sequences are provided in **Supplementary File 1**.

94

95 **Cell culture, transfection, and sorting**

HEK 293T cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum (FBS, Benchmarck), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were cultured in 75 cm^2 tissue culture flasks (NUNC, Thermo Scientific) at 37°C in a 5% CO_2 incubator. For each condition, a total of 10^6 cells/100 mm dish was cultured in triplicate overnight, then transfected with 1.93 pmol of GFP-expressing vectors and 3.47 pmol of gRNA vectors (**Supplementary Table 2**). Control cells received transfection reagents only. Transfections were conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h following transfection, the medium was replaced and cells were kept under culture for a total time of 48 h after transfection. Subsequently, cells were detached with EDTA, pelleted, washed twice, and resuspended in FACS buffer (Hank's balanced salt solution buffer supplemented with 1% BSA and 0.5 mM EDTA). Cell suspensions were then submitted to cell analysis and sorting in a FACSAria II cytometer (BD Biosciences). FACS data were analyzed using FACSDiva software (Becton Dickinson) with gating of single cells using FSC/W and SSC/W, and gating of GFP+ cells. When subsequent analyses were to be performed, cells were sorted into culture medium, washed twice with PBS, and pelleted.

CD34 FACS analysis

Cells were detached with EDTA, washed twice, and suspended in FACS buffer at 5 x 10^5 cells/mL. For each sample, three aliquots of 100 μL were prepared to be treated with CD34 PE monoclonal antibody (clone 4H11, eBioscience), isotype control PE Mouse IgG1 kappa (clone P3.6.2.8.1), and FACS buffer, respectively. Each aliquot was first treated with 20 μL of Fc receptor binding for 10 min on ice, then with 5 μL of PE antibody or buffer for 20 min on ice. After incubation, cells were washed (2 x 1 mL) and suspended in 500 μL of FACS buffer. FACS data were analyzed using FACSDiva (Becton Dickinson) or FloJow v10.5 (FlowJo LLC)

121 software, with gating of single cells using FSC/W and SSC/W, and gating of GFP+ and CD34
122 PE+ cells.

123

124 **Total RNA extraction and quantitative reverse-transcription polymerase chain reaction**
125 **(qRT-PCR)**

126 Cell pellets were treated with QIAzol lysis reagent (Qiagen) and total RNA was isolated using
127 the miRNAeasy kit (Quiagen) combined with DNAse (Qiagen) treatment according to
128 manufacturer's instructions. Synthesis of cDNA was performed with SuperScript III First-
129 Strand Synthesis System for RT-PCR (Life technologies) using random hexamers as primers.
130 *CD34*, *DDIT3*, *RELB*, and *JUNB* levels were measured with specific forward and reverse
131 primers (**Supplementary Table 3**) with Light Cycler 480 Syber Green Master mix, according
132 to the manufacturer's instructions.

133

134 **RNA-seq library preparation and analysis**

135 RNA-seq libraries were prepared from 1 ng of total RNA using the SMART-Seq HT Kit
136 (Takara) combined with Nextera XT kit (Illumina), according to manufacturers' instructions.
137 One-step cDNA synthesis and double-stranded cDNA amplification was conducted with 3'
138 SMART-Seq CDS Primer II A for priming, and SMART-Seq HT oligonucleotide for template
139 switching at the 5' end of the transcript. The cDNA was then purified with the Agencourt
140 AMPure XP kit, tagmented, and PCR amplified with appropriate index primers. Directional
141 RNA-seq libraries were then sequenced 100 bp single-end on the Illumina HiSeq 2500. Reads
142 were trimmed by Trim Galore
143 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; v0.3.7) and then aligned to
144 the hg38 reference genome using STAR v2.6.0c.[21] Differentially expressed protein-coding
145 genes were determined by applying a threshold of $\log_2$-fold change > 1, and FDR < 0.05, using
146 DESeq2 v1.16.1[22] on protein-coding gene counts normalized by housekeeping genes[23] as

input to the RUVg command within RUVseq v1.10.0.[24] A full description of the analysis can be found on our GitHub server: https://github.com/GreallyLab/Johnston_Simoes-Pires_et_al_2019.

Analysis of gene ontology enrichment and protein-protein associations

The list of 97 overlapping dysregulated genes was evaluated through functional enrichment analysis with DAVID (**Supplementary File 2**).[25] A total of 30 genes from enriched pathways showing a p-value < 0.005 were further analyzed for their predicted protein associations in the STRING database.[26]

Analysis of off-target effects

Predicted gRNA off-target sites were obtained from the CRISPOR website (http://crispor.tefor.net/crispor.py?batchId=0xd7m55fmDlcoF8EzTa9#s343+).[27] These regions were then intersected by +/- 1 kb from TSSs of the 97 overlapping dysregulated genes using *bedtools2* v2.26.0.

Determination of number of cell divisions

A total of 5 x 10^4 cells, either GFP+ (CRISPR CD34) or GFP- (control) were directly sorted into wells of a 24-well plate in culture medium. Cells were cultured and passaged every 48 h until GFP+ cells turned negative under the microscope. The total number of cells were counted at every passage, and the number of cell divisions was calculated as the population doubling level (PDL) with the formula $n = 3.32 (\log N_{24h} - \log N_0)$, where N_{24h} is the total number of cells after 24 h in culture, and N_0 is the number of cells seeded in the previous passage.

RESULTS

To investigate the VP16-based CRISPR activation system, we first designed a vector for the human expression of both a scrambled gRNA and the dCas9 fused to ten repeats of VP16 (VP160). In order to discriminate between transfected and non-transfected cells, green fluorescent protein (GFP) was fused to the VP160 open reading frame using a linker encoding the cleavable peptide 2A.[28] We used the system to target the endogenous activation of *CD34*, a gene which is not expressed in HEK 293 cells (https://www.proteinatlas.org).[29] *CD34* encodes a transmembrane protein, allowing us to discriminate easily by antibody recognition the cells expressing the protein in living cells. In a prior study, VP16 repeats directly fused to dCas9 required a pool of gRNAs for robust activation,[8] increasing the number of possible mismatches that could lead to off-target activation genome-wide. To test the off-target effects from multiple gRNA sequences, dCas9-VP160-2A-GFP was transfected in combination with six pooled gRNAs targeting the *CD34* promoter (**Fig. 1A**). Performing fluorescence-activated cell sorting (FACS), we demonstrated successfully induced endogenous expression of *CD34* in HEK 293T cells (**Fig. 1B**), with GFP+/CD34+ cells, showing an 80-fold increase in *CD34* mRNA levels (**Fig. 1C**). Interestingly, successfully transfected cells not expressing CD34 on the cell surface (GFP+/CD34-) also had an increase in *CD34* mRNA levels (**Fig. 1C**), suggesting a cell subpopulation with either delayed protein translation or a lack of membrane translocation. While the pooled gRNAs were indeed more effective in inducing CD34 trans-membrane expression compared to individual gRNAs, individual gRNA sequences seeding within a short distance (up to 100 nucleotides) from the transcriptional start site (TSS) were also successful, with expression levels increasing with decreasing distances from the TSS (**Supplementary Fig. 1**).

To evaluate whether the system induced undesirable effects genome-wide, we conducted RNA-seq analyses on the GFP+ cells transfected with the full activation system including the six gRNAs (CRISPR CD34), in comparison to non-transfected cells (Control). In addition to the strong upregulation of *CD34*, a total of 161 differentially expressed genes were identified (**Fig. 2A**). We then generated a CRISPR control by sorting GFP+ cells expressing dCas9-

198 VP160 and a scrambled gRNA (CRISPR). In this control, we detected 125 differentially
199 expressed genes (**Fig. 2B**), with 97 of them overlapping the genes identified in the CRISPR
200 CD34 sample (**Fig. 2C, Supplementary file 2**). Predicted gRNA off-target loci were not within
201 1kb of the dysregulated genes' TSSs, suggesting that their differential expression was not a
202 result of targeted dCas9-VP160 activation.

203 Nevertheless, the consistently dysregulated genes observed in the CRISPR control cells
204 raised the question of whether side effects may occur due to the expression of dCas9, VP16
205 repeats, or gRNAs. We evaluated these 97 genes through functional enrichment analysis and
206 protein associations. The gene ontology analysis was significantly enriched for biological
207 pathways related to apoptosis, response to cytokines, mechanical stimulus, inflammation, and
208 response to endoplasmic reticulum stress and unfolded proteins, represented by a total of 30
209 genes. Further analysis of protein-protein associations related to those genes featured the
210 pathways of cell defense and regulation of cell death (**Fig. 3**), from which we selected three
211 node genes (*DDIT3*, *RELB*, and *JUNB*) for further investigation.

212 *DDIT3* encodes the DNA Damage Inducible Transcript 3 transcription factor activated during
213 endoplasmic reticulum stress.[30] *RELB* is a subunit of the pleiotropic transcription factor NFκB
214 that has a central role in cell differentiation, growth, apoptosis, inflammation, and immunity.[31-
215 33] *JUNB*, a component of the AP1 transcription factor, has a role in stress response and is
216 associated with the NFκB pathway.[34-37]

217 Assessing the impact of the various CRISPR activation system components, we quantified the
218 changes in expression of the selected genes in GFP+ cells transfected with distinct CRISPR
219 components (**Fig. 4A**). Considering that the absolute amounts of foreign DNA introduced into
220 cells may contribute to the degree of the observed stress response, we used equimolar
221 plasmid concentrations across test conditions. First, we confirmed the activation of *CD34* in
222 the CRISPR CD34 cells only in the presence of the targeted gRNAs; it was not induced by the
223 expression of gRNAs alone (gRNA control) nor any other isolated component of the system
224 (**Fig. 4B**). The stress-related genes *DDIT3*, *RELB* and *JUNB* were induced across all samples

containing the CRISPR components. Expression of gRNAs in their untargeted form, either in the absence of dCas9 (gRNA control) or with a scrambled sequence in the presence of dCas9 (CRISPR and dCas9 controls), demonstrated a robust elevation of the stress-related genes' expression (**Fig. 4B**).

We then investigated whether cells transfected with the CRISPR activation system were able to return to their basal expression levels over multiple cell divisions. To do this, we kept the activated GFP+ cells in culture until cells were negative for GFP fluorescence under the microscope (after 10 cell divisions). At this point, the cells were analyzed by FACS and sorted for GFP- populations to ensure that the CRISPR components had been eliminated from the cells. We demonstrated that the upregulated stress-response genes returned to their basal levels (**Fig. 5**), indicating the absence of a memory effect for both *CD34* and the cellular stress response genes.

DISCUSSION

Taken together, our results point to the activation of stress genes as a side effect upon the expression of CRISPR components, especially untargeted gRNAs, not necessarily related to the presence of VP16 or to gRNA off-target sequences. Indeed, previous findings have shown that dCas9 has a higher residence time at a targeted genomic locus than at off-target loci,[38] potentially contributing to the high specificity of gRNAs in the dCas9-VP16-based epigenetic activation systems.

The outcome of undesirable transcriptional regulation is of concern when using dCas9 fused to effectors for epigenetic editing. The changes in cellular properties resulting from epigenetic editing might be expected to be heritable, as this is one definition of cellular epigenetic properties.[39] If side effects affecting gene expression are maintained through cell division, they will be difficult to uncouple from the desired effect of the epigenetic editing. Moreover,

heritable side effects may constitute a pitfall in developing CRISPR technologies for the development of therapeutic applications.

Our findings reveal an acute cellular response to the components of the CRISPR activation system, which dissipates over the course of multiple cell divisions. While this is reassuring for the use of CRISPR-mediated epigenetic editing, we note that the effects observed involve the transient activation of transcription factors. Transient upregulation of transcription factors may induce downstream pathways, which in turn can be irreversible. One example is the role of pioneer transcription factors in somatic cell reprogramming.[40] Accordingly, the transcription factor DDIT3, predominantly related to the stress response, has been identified as a regulatory node in erythroid lineage cell programming.[41] Furthermore, we only examined the genome-wide expression consequences of a transient CRISPR transfection in one cell line; the potential long-term transcriptional effects of stably transfected CRISPR machinery or differing cellular response by other cell types warrant further investigation.

CONCLUSION

An acute stress response occurs in cells when CRISPR components are used for gene activation. Although transient, the response was mediated through the upregulation of transcription factors that may, in certain cell systems, independently lead to reprogramming effects. Therefore, the impact of CRISPR components on transcription factors should be carefully taken into consideration when designing CRISPR genetic and epigenetic editing tools.

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DATA AVAILABILITY

All genome sequencing data are available from the NCBI Gene Expression Omnibus database under accession number GSE11827 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118277; reviewer token: ujijuqqqzlkjbyt).

CODE AVAILABILITY

The code files for the all analyses are available at https://github.com/GreallyLab/Johnston_Simoes-Pires_et_al_2019.

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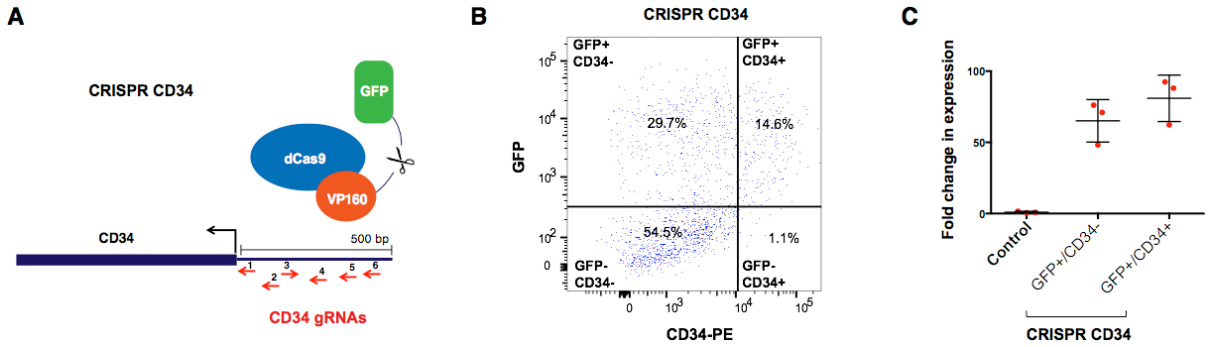


418

419 **Fig. 1.** Ectopic gene activation of *CD34* with the CRISPR activation system using the sgRNA-
420 dCas9-VP160-2A-GFP vector combined with 6 gRNAs. **A)** Overview of the CRISPR CD34
421 activation system: 6 gRNAs targeting the promoter of *CD34* within 500 bp from the TSS were
422 co-transfected with a vector expressing dCas9 fused to 10 repeats of the transactivation
423 peptide VP16, released from GFP by a cleavable peptide. **B)** FACS analysis of HEK 293 cells
424 transfected with the CRISPR CD34 activation system. GFP+/CD34- and GFP+/CD34+ cells
425 were sorted for subsequent analysis. **C)** The fold changes in expression in sorted cells relative
426 to control measured by qRT-PCR.

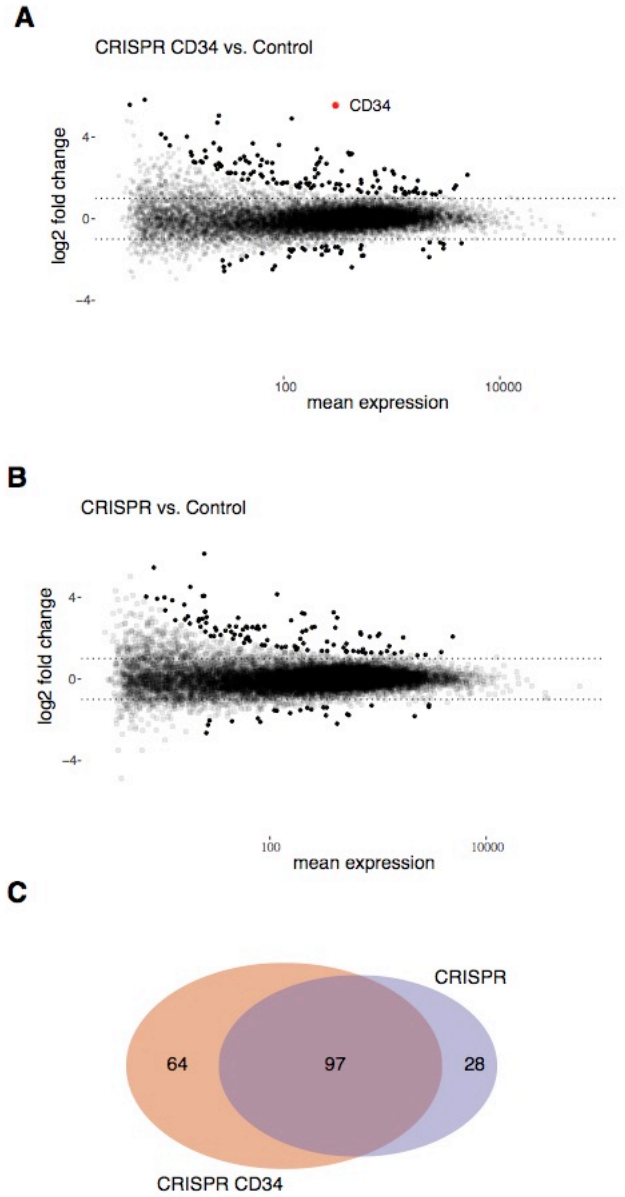
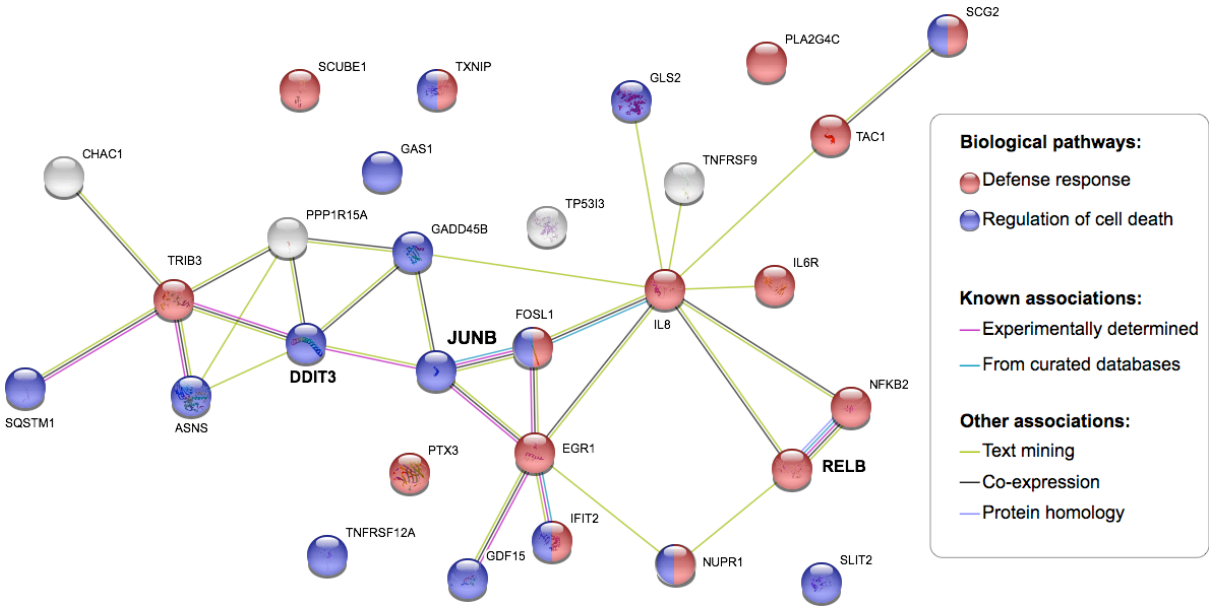


Fig. 2. Differentially expressed genes with the CRISPR CD34 activation system and the CRISPR system in the absence of targeted gRNAs. **A)** RNA-seq MA plot of CRISPR CD34 compared with control. Black solid dots are the differentially expressed genes (log_2 fold change > 1, FDR< 0.5). Differentially expressed *CD34* is represented by a solid red dot. **B)** RNA-seq MA plot of CRISPR control compared with control. Black solid dots are the differentially expressed genes (log_2 fold change > 2, FDR< 0.5A). *CD34* is not differentially expressed. **C)** Venn diagram representing the 97 differentially expressed genes in common between CRISPR CD34 and CRISPR control.



436

437 **Fig. 3. Protein-protein associations among genes selected from gene ontology**
438 **analysis.** Analysis from STRING database (https://string-db.org/). The genes *DDIT3*, *JUNB*
439 and *RELB* were selected for further studies as central to the regulation of these defense
440 response and cell death regulatory pathways.

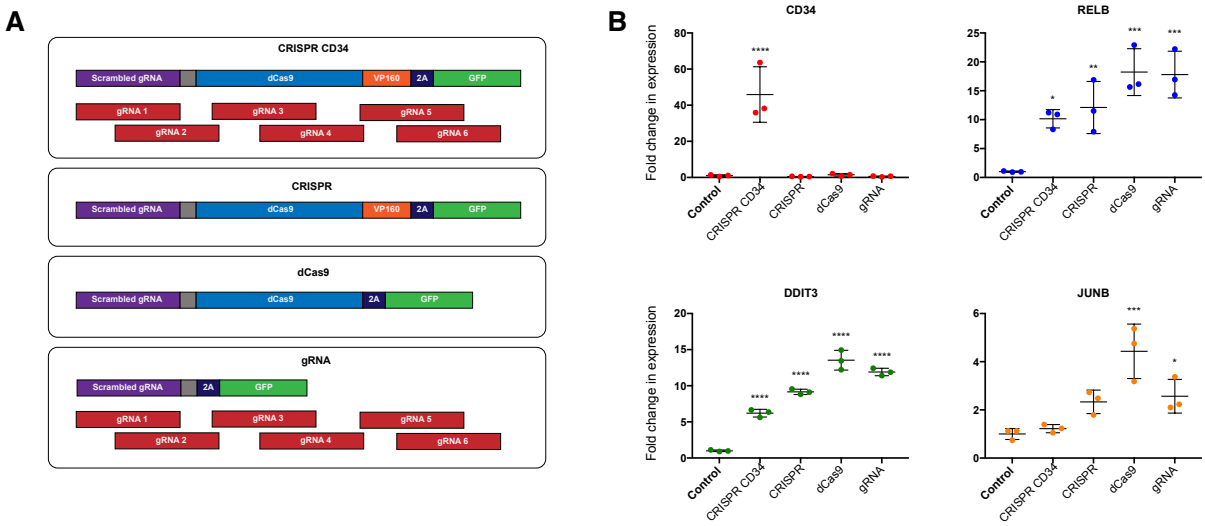


Fig 4. Relative RNA expression of *CD34* and stress-related genes across CRISPR conditions. **A)** s of the expression vectors transfected in each condition. **B)** Acute fold change in gene expression relative to control at 48 hours after transfection. P-values: *≤ 0.05; ***≤ 0.001; ****≤ 0.0001.

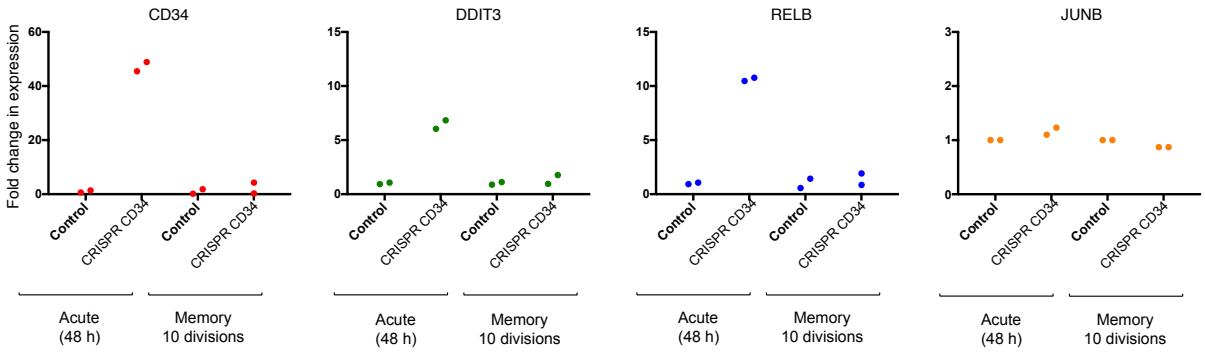
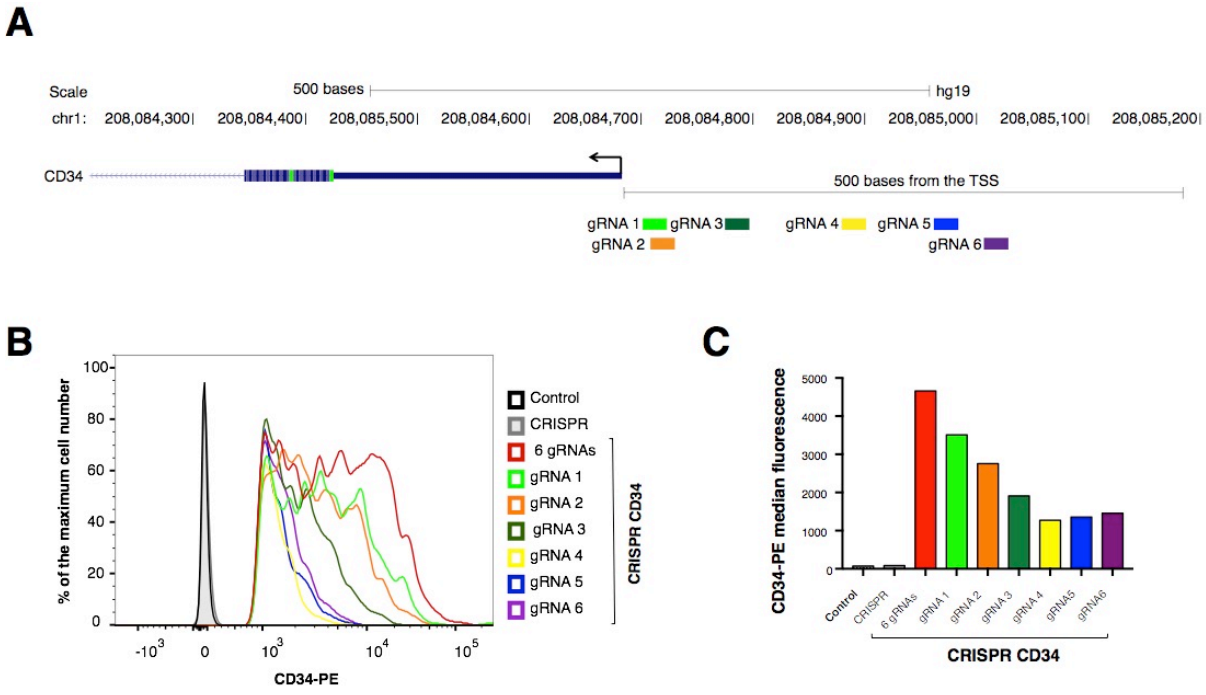


Fig. 5. Change in gene expression relative to control in transfected cells after 10 cell divisions in comparison to the acute response. The expected induction of gene expression is seen acutely at 48 h, with complete resolution when 10 cell divisions have occurred in these GFP- cells.



Supplementary Fig. 1. The efficiency of gRNA sequences in the activation of *CD34* using the VP16-based CRISPR activation system. A) The position of gRNAs 1-6 relative to the *CD34* transcriptional start site (TSS). **B)** FACS histograms depicting CD34 expression in cells transfected with the CRISPR activation system, followed by **C)** a bar graph depicting their median CD34-PE fluorescence.

Supplementary Table 1. gRNA sequences.

gRNA	Sequence	PAM	Distance to the TSS*
1	GAAAGCTGAACGAGGCATC	TGG	-19
2	CTCTCCAGAAAGCTGAACG	AGG	-26
3	CCGGCAAGGCTGCCACAAA	GGG	-93
4	CCTTTTGCAAGATTGTTAC	TGG	-197
5	CACTAAATGTGCCACATTG	TGG	-280
6	TGTGTGTGAGTGAAGCGTC	AGG	-324
Scramble	GGGTCTTCGAGAAGACCT	–	-

*TSS = transcriptional start site, defined as the first nucleotide in the gene transcript sequence including the UTR according to the Human Feb. 2009 (GRCh37/hg19) assembly (UCSC browser).

Supplementary Table 2. Amount of transfected vectors per 100 mm dishes across CRISPR conditions.

	Vector (pmol)				
Condition	sgRNA-dCas9-VP160-2A-GFP	sgRNA-dCas9-2A-GFP	sgRNA-2A-GFP	6 gRNAs	gRNA cloning vector (empty backbone)
CRISPR CD34	1.93	-	-	3.47*	-
CRISPR	1.93	-	-	-	3.47
dCas9	-	1.93	-	-	3.47
gRNA	-	-	1.93	3.47*	-

* Divided into equal amounts of each vector.

Supplementary Table 3. qRT PCR primers.

Gene	Forward primer	Reverse primer
CD34	AATAGCCAGTGATGCCCAAG	GGTATGCTCCCTGCTCCTT
DDIT3	GGAACCTGAGGAGAGAGTGTTC	TGCCATCTCTGCAGTTGGAT
RELB	CAGTGTGTGAGGAAGAAGGAG	CCGCAGCTCTGATGTGTTTGT
JUNB	CCACCTCCCGTTTACACCAA	GAGGTAGCTGATGGTGGTCG

Supplementary file 1

Sequence of plasmid sgRNA-dCas9-VP160-2A-GFP

U6 promoter

gRNA scaffold with scrambled target sequence

dCas9

VP160

2A peptide

GFP

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAAT

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Sequence of plasmid sgRNA-dCas9-2A-GFP

U6 promoter

gRNA scaffold with scrambled target sequence

dCas9

2A peptide

GFP

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746 CGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGT

Sequence of plasmid sgRNA-2A-GFP

U6 promoter

gRNA scaffold with scrambled target sequence

2A peptide

GFP

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818 TCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCT

819 GGCCTTTTGCTGGCCTTTTGCTCACATGT

820 **Supplementary file 2 (Excel file)**

821 DAVID enrichment analysis

Category	Term	Count	%	Pvalue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichm	Bonferroni	Benjamini	FDR
GOTERM_BP	GO:0043065~positive regulation of apoptotic process	9	9.27835052	**1.27E-04**	ENSG00000099860, ENSG0000000632	85	300	16792	5.92658824	0.08500249	0.08500249	0.19143515
GOTERM_BP	GO:0034097~response to cytokine	5	5.15463918	**1.31E-04**	ENSG00000160712, ENSG0000010485	85	52	16792	18.9954751	0.0877474	0.04488085	0.1979032
GOTERM_BP	GO:0070059~intrinsic apoptotic signaling pathway in response to endoplasmic reticu	4	4.12371134	**5.91E-04**	ENSG00000128965, ENSG0000017519	85	33	16792	23.9458111	0.33857494	0.12871504	0.88766902
GOTERM_BP	GO:0006954~inflammatory response	9	9.27835052	**6.12E-04**	ENSG00000105499, ENSG0000004924	85	379	16792	4.69123079	0.34806153	0.10143055	0.91854935
GOTERM_BP	GO:0036499~PERK-mediated unfolded protein response	3	3.09278351	**0.00157973**	ENSG00000175197, ENSG0000016942	85	12	16792	49.3882353	0.66882322	0.19829983	2.3555677
GOTERM_BP	GO:0009612~response to mechanical stimulus	4	4.12371134	**0.00320824**	ENSG00000265972, ENSG0000017559	85	59	16792	13.3934197	0.89419566	0.31227114	4.72957109
GOTERM_BP	GO:0042981~regulation of apoptotic process	6	6.18556701	**0.00429817**	ENSG00000049249, ENSG0000018044	85	213	16792	5.56487158	0.95075233	0.3495739	6.28821791
GOTERM_BP	GO:0034976~response to endoplasmic reticulum stress	4	4.12371134	0.00629307	ENSG00000175197, ENSG0000010125	85	75	16792	10.5361569	0.9878783	0.42396952	9.07953593
GOTERM_BP	GO:0032496~response to lipopolysaccharide	5	5.15463918	0.00923991	ENSG00000049249, ENSG0000000612	85	164	16792	6.02295552	0.99847951	0.51371916	13.0610902
GOTERM_BP	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	10	10.3092784	0.00974536	ENSG00000148677, ENSG0000013709	85	720	16792	2.74379085	0.99893568	0.49567869	13.7274392
GOTERM_BP	GO:0036101~leukotriene B4 catabolic process	2	2.06185567	0.01986165	ENSG00000186529, ENSG0000018611	85	4	16792	98.7764706	0.99999919	0.72051864	26.1018585
GOTERM_BP	GO:0032870~cellular response to hormone stimulus	3	3.09278351	0.02130019	ENSG00000145147, ENSG0000017122	85	45	16792	13.1701961	0.99999971	0.71467904	27.7203981
GOTERM_BP	GO:0006357~regulation of transcription from RNA polymerase II promoter	7	7.21649485	0.02311405	ENSG00000176046, ENSG0000010485	85	441	16792	3.13576097	0.99999992	0.71561133	29.7140548
GOTERM_BP	GO:0050900~leukocyte migration	4	4.12371134	0.02328278	ENSG00000168003, ENSG0000011541	85	122	16792	6.47714561	0.99999993	0.69155838	29.8968753
GOTERM_BP	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	11	11.3402062	0.02425333	ENSG00000148677, ENSG0000013709	85	981	16792	2.21517059	0.99999996	0.68150112	30.9398756
GOTERM_BP	GO:0071504~cellular response to heparin	2	2.06185567	0.02476584	ENSG00000145147, ENSG0000012073	85	5	16792	79.0211765	0.99999998	0.66565466	31.4847901
GOTERM_BP	GO:0035914~skeletal muscle cell differentiation	3	3.09278351	0.02497951	ENSG00000148677, ENSG0000017604	85	49	16792	12.095078	0.99999998	0.64659648	31.7107822
GOTERM_BP	GO:0001525~angiogenesis	5	5.15463918	0.02560005	ENSG00000087245, ENSG0000000632	85	223	16792	4.42943814	0.99999999	0.6347166	32.3631604
GOTERM_BP	GO:0007155~cell adhesion	7	7.21649485	0.02742974	ENSG00000008517, ENSG0000011541	85	459	16792	3.01278995	1	0.64056577	34.2530101
GOTERM_BP	GO:0007050~cell cycle arrest	4	4.12371134	0.03372607	ENSG00000175197, ENSG0000018044	85	141	16792	5.60433876	1	0.69852304	40.3863454
GOTERM_BP	GO:0001955~blood vessel maturation	2	2.06185567	0.03933341	ENSG00000087245, ENSG0000017519	85	8	16792	49.3882353	1	0.737022	45.3945927
GOTERM_BP	GO:0006690~icosanoid metabolic process	2	2.06185567	0.03933341	ENSG00000186529, ENSG0000018611	85	8	16792	49.3882353	1	0.737022	45.3945927
GOTERM_BP	GO:0060337~type I interferon signaling pathway	3	3.09278351	0.04081791	ENSG00000119922, ENSG0000018760	85	64	16792	9.26029412	1	0.73395973	46.6531134
GOTERM_BP	GO:0071347~cellular response to interleukin-1	3	3.09278351	0.04920556	ENSG00000148677, ENSG0000012425	85	71	16792	8.34730737	1	0.78421352	53.2699592
GOTERM_BP	GO:0007568~aging	4	4.12371134	0.04983591	ENSG00000149131, ENSG0000007715	85	165	16792	4.78916221	1	0.77437635	53.7349041
GOTERM_BP	GO:0035556~intracellular signal transduction	6	6.18556701	0.05144493	ENSG00000105499, ENSG0000013709	85	403	16792	2.94123486	1	0.77161432	54.9022078
GOTERM_BP	GO:0010955~negative regulation of protein processing	2	2.06185567	0.05368593	ENSG00000128965, ENSG0000018044	85	11	16792	35.9187166	1	0.77316077	56.4822284
GOTERM_BP	GO:2000010~positive regulation of protein localization to cell surface	2	2.06185567	0.05842286	ENSG00000144452, ENSG0000016690	85	12	16792	32.9254902	1	0.78954538	59.6534515
GOTERM_BP	GO:0001878~response to yeast	2	2.06185567	0.06313636	ENSG00000006128, ENSG0000016366	85	13	16792	30.3927602	1	0.80369959	62.5937505
GOTERM_BP	GO:0045087~innate immune response	6	6.18556701	0.06433245	ENSG00000006128, ENSG0000010485	85	430	16792	2.75655267	1	0.79865993	63.3073757
GOTERM_BP	GO:0009636~response to toxic substance	3	3.09278351	0.06762261	ENSG00000176046, ENSG0000015101	85	85	16792	6.97245675	1	0.80434661	65.2053613
GOTERM_BP	GO:0014912~negative regulation of smooth muscle cell migration	2	2.06185567	0.06782654	ENSG00000145147, ENSG0000011546	85	14	16792	28.2218487	1	0.79478903	65.3199289
GOTERM_BP	GO:0006691~leukotriene metabolic process	2	2.06185567	0.07249352	ENSG00000186529, ENSG0000018611	85	15	16792	26.3403922	1	0.8067677	67.8475679
GOTERM_BP	GO:0050930~induction of positive chemotaxis	2	2.06185567	0.07249352	ENSG00000171951, ENSG0000016942	85	15	16792	26.3403922	1	0.8067677	67.8475679
GOTERM_BP	GO:0030198~extracellular matrix organization	4	4.12371134	0.07519545	ENSG00000187955, ENSG0000011541	85	196	16792	4.03169268	1	0.80906902	69.2312109
GOTERM_BP	GO:0010466~negative regulation of peptidase activity	2	2.06185567	0.08175832	ENSG00000197558, ENSG0000014725	85	17	16792	23.2415225	1	0.8268432	72.3639712
GOTERM_BP	GO:0071456~cellular response to hypoxia	3	3.09278351	0.08343126	ENSG00000148677, ENSG0000007823	85	96	16792	6.17352941	1	0.82445767	73.1134738
GOTERM_BP	GO:0042267~natural killer cell mediated cytotoxicity	2	2.06185567	0.08635637	ENSG00000153879, ENSG0000011198	85	18	16792	21.9503268	1	0.82685228	74.3785565
GOTERM_BP	GO:0045926~negative regulation of growth	2	2.06185567	0.09093167	ENSG00000147257, ENSG0000011546	85	19	16792	20.7950464	1	0.83487604	76.2463919
GOTERM_BP	GO:0002576~platelet degranulation	3	3.09278351	0.09402062	ENSG00000104112, ENSG0000011541	85	103	16792	5.75396916	1	0.83736925	77.4346813
GOTERM_BP	GO:0006541~glutamine metabolic process	2	2.06185567	0.09548433	ENSG00000135423, ENSG0000007066	85	20	16792	19.7552941	1	0.8344831	77.9781594
GOTERM_BP	GO:0001649~osteoblast differentiation	3	3.09278351	0.09556404	ENSG00000136235, ENSG0000017122	85	104	16792	5.69864253	1	0.82713684	78.0074014
GOTERM_BP	GO:0051091~positive regulation of sequence-specific DNA binding transcription facto	3	3.09278351	0.09711478	ENSG00000175592, ENSG0000013316	85	105	16792	5.64436975	1	0.82477787	78.5691492

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824 Enriched pathways

GO:0043065~positive regulation of apoptotic process	
ENSG00000175592	FOS like 1, AP-1 transcription factor subunit(FOSL1)
ENSG00000006327	TNF receptor superfamily member 12A(TNFRSF12A)
ENSG00000148677	ankyrin repeat domain 1(ANKRD1)
ENSG00000099860	growth arrest and DNA damage inducible beta(GADD45B)
ENSG00000119922	interferon induced protein with tetratricopeptide repeats 2(IFIT2)
ENSG00000176046	nuclear protein 1, transcriptional regulator(NUPR1)
ENSG00000161011	sequestosome 1(SQSTM1)
ENSG00000145147	slit guidance ligand 2(SLIT2)
ENSG00000265972	thioredoxin interacting protein(TXNIP)
GO:0034097~response to cytokine	
ENSG00000175592	FOS like 1, AP-1 transcription factor subunit(FOSL1)
ENSG00000171223	JunB proto-oncogene, AP-1 transcription factor subunit(JUNB)
ENSG00000104856	RELB proto-oncogene, NF-kB subunit(RELB)
ENSG00000160712	interleukin 6 receptor(IL6R)
ENSG00000077150	nuclear factor kappa B subunit 2(NFKB2)
GO:0070059~intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	
ENSG00000128965	ChaC glutathione specific gamma-glutamylcyclotransferase 1(CHAC1)
ENSG00000175197	DNA damage inducible transcript 3(DDIT3)
ENSG00000087074	protein phosphatase 1 regulatory subunit 15A(PPP1R15A)
ENSG00000101255	tribbles pseudokinase 3(TRIB3)
GO:0006954~inflammatory response	
ENSG00000169429	C-X-C motif chemokine ligand 8(CXCL8)
ENSG00000104856	RELB proto-oncogene, NF-kB subunit(RELB)
ENSG00000049249	TNF receptor superfamily member 9(TNFRSF9)
ENSG00000077150	nuclear factor kappa B subunit 2(NFKB2)
ENSG00000163661	pentraxin 3(PTX3)
ENSG00000105499	phospholipase A2 group IVC(PLA2G4C)
ENSG00000171951	secretogranin II(SCG2)
ENSG00000159307	signal peptide, CUB domain and EGF like domain containing 1(SCUBE1)
ENSG00000006128	tachykinin precursor 1(TAC1)
GO:0036499~PERK-mediated unfolded protein response	
ENSG00000169429	C-X-C motif chemokine ligand 8(CXCL8)
ENSG00000175197	DNA damage inducible transcript 3(DDIT3)
ENSG00000070669	asparagine synthetase (glutamine-hydrolyzing)(ASNS)
GO:0009612~response to mechanical stimulus	
ENSG00000175592	FOS like 1, AP-1 transcription factor subunit(FOSL1)
ENSG00000171223	JunB proto-oncogene, AP-1 transcription factor subunit(JUNB)
ENSG00000070669	asparagine synthetase (glutamine-hydrolyzing)(ASNS)
ENSG00000265972	thioredoxin interacting protein(TXNIP)
GO:0042981~regulation of apoptotic process	
ENSG00000049249	TNF receptor superfamily member 9(TNFRSF9)
ENSG00000120738	early growth response 1(EGR1)
ENSG00000135423	glutaminase 2(GLS2)
ENSG00000180447	growth arrest specific 1(GAS1)
ENSG00000130513	growth differentiation factor 15(GDF15)
ENSG00000115129	tumor protein p53 inducible protein 3(TP53I3)

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827 David total 30 genes

ENSG00000175592	FOS like 1, AP-1 transcription factor subunit(FOSL1)	FOSL1
ENSG00000006327	TNF receptor superfamily member 12A(TNFRSF12A)	TNFRSF12A
ENSG00000148677	ankyrin repeat domain 1(ANKRD1)	NKRD1
ENSG00000099860	growth arrest and DNA damage inducible beta(GADD45B)	GADD45B
ENSG00000119922	interferon induced protein with tetratricopeptide repeats 2(IFIT2)	IFIT2
ENSG00000176046	nuclear protein 1, transcriptional regulator(NUPR1)	NUPR1
ENSG00000161011	sequestosome 1(SQSTM1)	SQSTM1
ENSG00000145147	slit guidance ligand 2(SLIT2)	SLIT2
ENSG00000265972	thioredoxin interacting protein(TXNIP)	TXNIP
ENSG00000171223	JunB proto-oncogene, AP-1 transcription factor subunit(JUNB)	JUNB
ENSG00000104856	RELB proto-oncogene, NF-kB subunit(RELB)	RELB
ENSG00000160712	interleukin 6 receptor(IL6R)	IL6R
ENSG00000077150	nuclear factor kappa B subunit 2(NFKB2)	NFKB2
ENSG00000128965	ChaC glutathione specific gamma-glutamylcyclotransferase 1(CHAC1)	CHAC1
ENSG00000175197	DNA damage inducible transcript 3(DDIT3)	DDIT3
ENSG00000087074	protein phosphatase 1 regulatory subunit 15A(PPP1R15A)	PPP1R15A
ENSG00000101255	tribbles pseudokinase 3(TRIB3)	TRIB3
ENSG00000169429	C-X-C motif chemokine ligand 8(CXCL8)	CXCL8
ENSG00000049249	TNF receptor superfamily member 9(TNFRSF9)	TNFRSF9
ENSG00000163661	pentraxin 3(PTX3)	PTX3
ENSG00000105499	phospholipase A2 group IVC(PLA2G4C)	PLA2G4C
ENSG00000171951	secretogranin II(SCG2)	SCG2
ENSG00000159307	signal peptide, CUB domain and EGF like domain containing 1(SCUBE1)	SCUBE1
ENSG00000006128	tachykinin precursor 1(TAC1)	TAC1
ENSG00000070669	asparagine synthetase (glutamine-hydrolyzing)(ASNS)	ASNS
ENSG00000120738	early growth response 1(EGR1)	EGR1
ENSG00000135423	glutaminase 2(GLS2)	GLS2
ENSG00000180447	growth arrest specific 1(GAS1)	GAS1
ENSG00000130513	growth differentiation factor 15(GDF15)	GDF15
ENSG00000115129	tumor protein p53 inducible protein 3(TP53I3)	TP53I3

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