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**Identification of hyperosmotic stress-responsive genes in Chinese hamster ovary cells
via genome-wide virus-free CRISPR/Cas9 screening**

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1 **Abstract**

2 Chinese hamster ovary (CHO) cells are the preferred mammalian host cells for therapeutic protein
3 production that have been extensively engineered to possess the desired attributes for high-yield
4 protein production. However, empirical approaches for identifying novel engineering targets are
5 laborious and time-consuming. Here, we established a genome-wide CRISPR/Cas9 screening platform
6 for CHO-K1 cells with 111,651 guide RNAs (gRNAs) targeting 21,585 genes using a virus-free
7 recombinase-mediated cassette exchange-based gRNA integration method. Using this platform, we
8 performed a positive selection screening under hyperosmotic stress conditions and identified 180
9 genes whose perturbations conferred resistance to hyperosmotic stress in CHO cells. Functional
10 enrichment analysis identified hyperosmotic stress responsive gene clusters, such as tRNA wobble
11 uridine modification and signaling pathways associated with cell cycle arrest. Furthermore, we
12 validated 32 top-scoring candidates and observed a high rate of hit confirmation, demonstrating the
13 potential of the screening platform. Knockout of the novel target genes, *Zfr* and *Pnp*, in monoclonal
14 antibody (mAb)-producing recombinant CHO (rCHO) cells and bispecific antibody (bsAb)-producing
15 rCHO cells enhanced their resistance to hyperosmotic stress, thereby improving mAb and bsAb
16 production. Overall, the collective findings demonstrate the value of the screening platform as a
17 powerful tool to investigate the functions of genes associated with hyperosmotic stress and to
18 discover novel targets for rational cell engineering on a genome-wide scale in CHO cells.

19

20 **Keywords:** Chinese hamster ovary cell, CRISPR/Cas9 screen, Genome-wide screen, Osmotic stress,
21 Therapeutic protein

22

1 **Abbreviations:** bsAb, bispecific antibody; Cas9, CRISPR-associated protein 9; CHO, Chinese hamster
2 ovary; CRISPR, clustered regularly interspaced short palindromic repeats; FACS, fluorescence-
3 activated cell sorting; GO, Gene ontology; gRNA, guide RNA; LP, landing pad; mAb, monoclonal
4 antibody; MCL, master cell line; NGS, next-generation sequencing; PCR, polymerase chain reaction;
5 qRT-PCR, quantitative real-time polymerase chain reaction; RMCE, recombinase-mediated cassette
6 exchange

7

8 **1. Introduction**

9 Chinese hamster ovary (CHO) cells have been widely used for the large-scale production of
10 recombinant biotherapeutics (Walsh, 2018; Wurm, 2013). Extensive efforts have been made in CHO
11 cell engineering to achieve high-yield production with improved product quality and low
12 manufacturing costs (Kim et al., 2012; Tihanyi and Nyitray, 2021). However, as current cell engineering
13 strategies mostly rely on known targets, new strategies are required to identify novel targets in CHO
14 cells.

15 Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9
16 (Cas9) technology has facilitated high-throughput large-scale functional screening, which can be used
17 to identify novel targets associated with a specific phenotype of interest (Shalem et al., 2014; Wang
18 et al., 2014). Being a powerful tool for gene perturbations, CRISPR screens have been widely used in
19 various applications, such as investigation of molecular and cellular biology, genetic disease, cancer,
20 and microbial engineering (Bock et al., 2022). Recently, a metabolic CRISPR/Cas9 screen in CHO cells
21 identified a novel target, whose deletion improved cell growth in glutamine-free media (Karottki et
22 al., 2021). Therefore, CRISPR genetic screening is a promising tool to discover novel targets for CHO

1 cell engineering.

2 Most pooled CRISPR screens rely on lentivirus-based delivery methods for the introduction of guide
3 RNAs (gRNAs) (Joung et al., 2017). However, working with lentiviruses requires advanced biosafety
4 facilities, which may be cumbersome in terms of technical accessibility. Despite delicate adjustment
5 of the multiplicity of infection, transducing viruses can cause no or multiple integrations of gRNA,
6 following a Poisson distribution, which can decrease the signal-to-noise ratio (Ellis and Delbrück,
7 1939). To overcome these drawbacks, alternative approaches using plasmid transfection, such as
8 Cas9-mediated homologous recombination (Rajagopal et al., 2016), recombinase-mediated cassette
9 exchange (RMCE) (Viswanatha et al., 2018), and transposons (Chang et al., 2020) have provided new
10 options to replace lentivirus-based methods. Notably, RMCE coupled with a landing pad platform,
11 which encourages single gene integration into a pre-defined site, shows a higher efficiency of single-
12 copy gRNA integration with minimized clonal variation compared to the lentivirus-based system
13 (Xiong et al., 2021).

14 For the large-scale production of recombinant biotherapeutics, including monoclonal antibodies
15 (mAbs), fed-batch culture supporting high volumetric productivity is widely used because of its
16 operational simplicity and reliability (Fike, 2009). In fed-batch cultures, culture osmolality increases
17 with culture time due to repeated feeding of nutrient concentrates and addition of a base to maintain
18 optimal pH during culture, which induces apoptotic cell death (Han et al., 2010). Hence, the use of an
19 apoptosis-resistant CHO cell line can further increase the volumetric productivity of recombinant
20 biotherapeutics in fed-batch cultures by extending the culture duration. Recently, CRISPR/Cas9
21 screening using gRNA libraries targeting genes related to kinase and cell cycle identified an apoptosis-
22 related target whose deletion conferred resistance to osmotic stress in rHEK293 cells (Shin et al.,

1 2022). However, little is known about the functional characterization of genes related to
2 hyperosmotic conditions in CHO cells on a genome-wide scale.

3 In this study, we performed genome-wide CRISPR knockout screening of CHO-K1 cells using a virus-
4 free RMCE-based gRNA integration method to identify novel genes associated with hyperosmotic
5 stress. A proliferation-based positive-selection screen was conducted against hyperosmotic stress
6 conditions and genes that were targeted by significantly enriched gRNAs were identified. Functional
7 enrichment analysis was conducted using the identified genes and hyperosmotic stress responsive
8 gene networks were elucidated. Perturbations of the 32 highest-ranking genes, whose gRNAs showed
9 significant enrichment on the screen, were validated in CHO-K1 cells. We focused on *Zfr* and *Pnp*
10 genes and verified their perturbations in mAb- and bispecific antibody (bsAb)-producing recombinant
11 CHO (rCHO) cell lines.

12

13 **2. Materials and methods**

14 *2.1. Cloning and plasmid constructions*

15 All plasmids used in this study are listed in Supplementary Table S1. A TagBFP RMCE donor plasmid
16 and nuclear localization signal (NLS)-Bxb1 recombinase plasmid were constructed using the uracil-
17 specific excision reagent (USER) cloning method, as previously described (Lee et al., 2015). TagBFP
18 RMCE donor plasmid was generated from the attB-Puro-U6-gRNA-attB^{mut} plasmid (Xiong et al., 2021).
19 TagBFP-coding sequence was amplified from the plasmid described previously (Sergeeva et al., 2020).
20 NLS-Bxb1 recombinase plasmid was generated from the PSF-CMV-Bxb1 recombinase plasmid (Xiong
21 et al., 2021). NLS sequences were included in the USER primers to be attached to both the N- and C-

1 termini of Bxb1 recombinase. All primers used for cloning are listed in Supplementary Table S2. To
2 generate an all-in-one CRISPR/Cas9 plasmid for screening verification, the annealed gRNA oligos were
3 cloned into the BbsI site of the pSpCas9(BB)-2A-BSD plasmid (Addgene plasmid # 118055; a gift from
4 Ken-Ichi Takemaru) using T4 ligase, according to the manufacturer's instructions. All gRNAs used in
5 this study are listed in Supplementary Table S3. All constructs were verified by sequencing and
6 purified using a NucleoBond Xtra Midi EF kit (Macherey-Nagel, Düren, Germany), according to the
7 manufacturer's instructions.

8

9 *2.2. Library design and construction*

10 To construct a CHO-K1 genome-wide CRISPR knockout library, we designed 111,651 unique gRNAs
11 against 21,585 genes. The library was designed and constructed as previously described (Xiong et al.,
12 2021). DESKTOP Genetics (DESKGEN, London, UK) designed a whole-genome gRNA library against the
13 Chinese hamster PICR scaffold. All protein-coding gene IDs were independently targeted. Ideally, five
14 gRNAs were designed per gene that preferentially targeted the predicted functional domains.
15 Additionally, 1000 non-targeting gRNAs were designed in the library as internal controls. This resulted
16 in a total library of 108,580 gRNAs targeting 21,585 genes. To precisely target the CHO-K1 cell line, it
17 was necessary to correct the library design. With a large number of Illumina reads from CHO samples
18 at Denmark Technical University, it was possible to overlay CHO reads on the PICR scaffold and identify
19 the mutations present in CHO-K1 cells. Mutations were categorized as indels and single nucleotide
20 polymorphisms. If gRNAs bound to regions with mutations, the gRNA sequence was corrected to
21 target the mutated sequence. This resulted in the selection of 3,071 gRNAs. For convenience, the
22 corrected gRNAs for CHO-K1 genome were added directly to the library. Therefore, a final library

1 consisting of 111,651 gRNAs targeting 21,585 genes was designed. The designed library was
2 synthesized as an oligo by Twist Bioscience (San Francisco, CA).

3

4 *2.3. Cell lines, culture maintenance, and culture media*

5 CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY)
6 supplemented with 7% fetal bovine serum (HyClone, Logan, UT). Cells were maintained in T25 flasks
7 at 37°C with 5% CO₂ and passaged every three days. CHO-K1 cells were adapted to grow in suspension
8 culture in 125 mL Erlenmeyer flasks (Corning, Corning, NY) containing 30 mL of CD CHO medium
9 (Gibco) supplemented with 4 mM glutamine (HyClone) and 100X anti-clumping agent (Lonza, Basel,
10 Switzerland) in a climo-shaking CO₂ incubator (ISF1-X; Adolf Kuhner AG, Birsfelden, Switzerland) at
11 110 rpm, 37 °C, 5% CO₂, and 85% humidity. CHO-K1 cell line producing rituximab (CHO-mAb) was
12 established as previously described (Park et al., 2016). CHO-mAb cell line was maintained in
13 PowerCHO2CD medium (Lonza) supplemented with glutamine synthetase expression medium
14 (Sigma-Aldrich, St. Louis, MO), and 25 μM methionine sulfoximine (Sigma-Aldrich) in 125 mL
15 Erlenmeyer flasks. CHO-S cell line producing bsAb (CHO-bsAb) was provided by ABL Bio (Gyeonggi-
16 Do, Korea). CHO-bsAb cell line was maintained in Dynamis medium (Gibco) supplemented with 4 mM
17 glutamine, 100 nM methotrexate (Sigma-Aldrich), and 0.2% anti-clumping agent (Thermo Fisher
18 Scientific, Waltham, MA). Viable cells were distinguished from dead cells using the trypan blue dye
19 exclusion method, and cell concentration was estimated using a Countess II FL automated cell counter
20 (Invitrogen, Carlsbad, CA).

21

1 *2.4. Generation of the landing pad master cell line (MCL) using the CRISPR/Cas9-based RMCE landing*
2 *pad platform*

3 CHO-K1 MCL was generated as previously described (Grav et al., 2018). CHO-K1 cells (0.5×10^6
4 cells/mL) were seeded in T25 flasks. After 24 h, cells were transfected with an LP donor plasmid, a
5 gRNA plasmid targeting the non-coding region (site T2), and a Cas9 plasmid at a ratio of 1:1:1 (w/w)
6 using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. To generate
7 stable cell pools, 800 $\mu\text{g}/\text{mL}$ hygromycin (Clontech, San Jose, CA) was used for the selection. The
8 medium was changed every three days. After two weeks of selection, mCherry-positive/ZsGreen1-
9 negative cell pools were sorted using FACS Aria II (BD Biosciences, San Jose, CA) with a 488 nm blue
10 laser and 530/30 and 610/20 filters. Subsequently, stable cell lines were generated using limiting
11 dilution method to a concentration of 0.3 cell/well into a 96-well plate. Clones were expanded and
12 verified by 5'/3'-junction polymerase chain reaction (PCR), copy number, mRNA expression, and
13 fluorescence level analyses (Supplementary Fig. S1).

14

15 *2.5. Generation of a cell-based gRNA library*

16 To ensure the coverage of 500 cells per gRNA, which can ensure sufficient representation of the gRNA
17 library (Doench, 2018; Joung et al., 2017), the number of cells required for transfection was calculated
18 based on the measured value of RMCE efficiency, which was 10.5% (Supplementary Fig. S2). One day
19 before transfection, a total of 3.0×10^8 cells were seeded at 0.5×10^6 cells/mL in three 500 mL
20 Erlenmeyer flasks (Corning) containing 200 mL of CD-CHO supplemented with 4 mM glutamine. On
21 the day of transfection, cells were reseeded at 1.0×10^6 cells/mL in twelve 125 mL Erlenmeyer flasks
22 containing 50 mL of the medium. Cells were then transfected with the gRNA library and NLS-Bxb1

1 recombinase plasmids at a ratio of 3:1 (w/w) using a FreeStyle Max transfection reagent (Thermo
2 Fisher), according to the manufacturer's instructions. One day after transfection, an anti-clumping
3 agent was added to the cells. Two days after transfection, cells were combined and sub-cultured into
4 four 500 mL Erlenmeyer flasks containing 250 mL of the medium. Three days after transfection, cells
5 were treated with 10 µg/mL puromycin (Sigma-Aldrich). Cell pools were passaged every three days
6 with 10 µg/mL puromycin treatment. Fourteen days after transfection, recovered cell pools were
7 subjected to Cas9 transfection, and a total of 5.6×10^7 cells were used for genomic DNA extraction.

8

9 *2.6. Generation of a knockout library cell pool*

10 To ensure sufficient coverage, the number of cells required for transfection was calculated based on
11 the measured Cas9 transfection efficiency, which was 20.4% (Supplementary Fig. S3). One day before
12 transfection, a total of 3.0×10^8 cells were seeded at 0.5×10^6 cells/mL in three 500 mL Erlenmeyer
13 flasks containing 200 mL of CD-CHO supplemented with 4 mM glutamine. On the day of transfection,
14 cells were reseeded at 1.0×10^6 cells/mL in twelve 125 mL Erlenmeyer flasks containing 50 mL of the
15 medium. Cells were then transfected with a Cas9-BSD plasmid using the FreeStyle Max transfection
16 reagent (Thermo Fisher), according to the manufacturer's instructions. One day after transfection,
17 cells were treated with 10 µg/mL blasticidin (Sigma-Aldrich). Two days after transfection, the medium
18 was replaced with a fresh medium containing 10 µg/mL blasticidin. Four days after transfection, cells
19 were recovered in a medium without blasticidin. Eight and sixteen days after transfection, a total of
20 5.6×10^7 cells were prepared for genomic DNA extraction.

21

1 *2.7. Hyperosmotic stress screening*

2 To prepare a hyperosmolar medium (463 ± 4 mOsm/kg), 1.8 mL of 5 M NaCl (Sigma-Aldrich) was
3 added to 118.2 mL of the standard medium (325 ± 1 mOsm/kg), which was the CD-CHO supplemented
4 with 4 mM glutamine and 100X anti-clumping agent. Osmolality was measured using the Fiske Micro-
5 Osmometer (Thermo Fisher Scientific). To sufficiently cover 500 cells per gRNA, a total of 6.0×10^7
6 cells were seeded in triplicate at 0.5×10^6 cells/mL in 500 mL Erlenmeyer flasks containing 120 mL of
7 the standard or hyperosmolar medium and passaged every three days. After 21 days, a total of $5.6 \times$
8 10^7 cells were used for genomic DNA extraction.

9

10 *2.8. Preparation of next-generation sequencing (NGS) samples*

11 Genomic DNA samples were extracted using the Exgene Blood SV kit (GeneAll Biotechnology, Seoul,
12 South Korea), according to the manufacturer's instructions. To prepare NGS samples, PCR was
13 performed in a total volume of 50 μ L with 4.0 μ g genomic DNA per reaction using NEBNext Ultra II
14 Q5 Master Mix (New England Biolabs, Ipswich, MA) (98 °C for 3 min; 22 cycles: 98 °C for 10 s, 60 °C
15 for 30 s, 72 °C for 30 s; 72 °C for 5 min) using primers listed in Supplementary Table S2. PCR products
16 were purified using a NucleoSpin Gel and PCR purification kit (Macherey-Nagel) and indexed using a
17 TruSeq Nano DNA Library Prep kit (Illumina, San Diego, CA). The resulting library was quantified with
18 a Qubit Flex Fluorometer (Thermo Fisher Scientific) using a dsDNA HS Assay kit (Thermo Fisher
19 Scientific). Fragment size was determined using a 2100 Bioanalyzer Instrument (Agilent, Santa Clara,
20 CA) and TapeStation D5000 (Agilent) and sequenced on a NextSeq 500 sequencer or a NextSeq 550
21 sequencer (Illumina).

1

2 *2.9. NGS data analysis*

3 For the analysis of gRNA fold-changes, raw FASTQ files were analyzed using Model-based Analysis of
4 Genome-wide CRISPR/Cas9 Knockout (MAGeCK) (Li et al., 2014) and Platform-independent Analysis
5 of Pooled screens using Python (PinAPL-Py) (<http://pinapl-py.ucsd.edu/>) (Spahn et al., 2017).
6 MAGeCK v0.5.9.5 was run following the instructions
7 (<https://sourceforge.net/p/mageck/wiki/Home/#usage>). The gene-test-fdr-threshold parameter was
8 set to 0.01, and the other parameters were set to default. PinAPL-Py v2.9 was run using an adjusted
9 robust rank aggregation (α RRA) ranking metric, an `fdr_hb` p-value adjustment method, and other
10 default parameters. Top candidates for enriched gRNAs were ranked using the α RRA method and
11 filtered using a p-value threshold of 0.01. A gene was considered to be “significant” if it was
12 statistically significant at the gRNA level for at least half of the gRNAs from the total designed gRNAs
13 targeting the gene.

14

15 *2.10. Functional enrichment analysis*

16 Gene ontology (GO) enrichment analysis was performed using the Database for Annotation,
17 Visualization, and Integrated Discovery (DAVID) Knowledgebase v2022q2 (<https://david.ncifcrf.gov/>)
18 (Huang da et al., 2009; Sherman et al., 2022). Pathway and process enrichment analyses were carried
19 out using ontology resources from GO biological processes (Ashburner et al., 2000), Kyoto
20 Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000), Reactome Gene Sets (Fabregat et
21 al., 2018), and Molecular Signatures Database (Subramanian et al., 2005) using Metascape

1 (<https://metascape.org/>) (Zhou et al., 2019). The network was visualized using Cytoscape v3.9.1
2 (Shannon et al., 2003).

3

4 *2.11. Generation of knockout cell pools using all-in-one CRISPR/Cas9 plasmids*

5 To generate a knockout cell pool in CHO-K1 cells, MCL was seeded at 1×10^6 cells/mL in a 12-well
6 plate containing 1 mL CD-CHO supplemented with 4 mM glutamine and transfected with all-in-one
7 CRISPR/Cas9 plasmids targeting each gene using FreestyleMax, according to the manufacturer's
8 instructions. After 48 h, transfected cells were treated with 150 $\mu\text{g}/\text{mL}$ blasticidin for three days and
9 recovered for nine days without blasticidin. To generate knockout cell pools in CHO-mAb and CHO-
10 bsAb cell lines, cells were seeded at 1×10^6 cells/mL in a 6-well plate containing 3 mL SFM4Transfx-
11 293 (HyClone) supplemented with 4 mM glutamine and transfected with all-in-one CRISPR/Cas9
12 plasmids targeting each gene using FreestyleMax according to the manufacturer's instructions. After
13 48 h, transfected cells were treated with 75 $\mu\text{g}/\text{mL}$ blasticidin for CHO-mAb and 150 $\mu\text{g}/\text{mL}$ blasticidin
14 for CHO-bsAb cell lines. After three days of blasticidin treatment, cells were recovered for nine days
15 without blasticidin treatment.

16

17 *2.12. Batch culture*

18 CHO-K1 knockout cell pools were seeded at 0.5×10^6 cells/mL in a 12-well plate containing 1 mL of
19 the hyperosmolar medium used for screening and incubated at 37 °C, 85 % humidity, 5 % CO₂, and
20 110 rpm. Cell viability and concentration were measured every two days. For CHO-mAb and CHO-
21 bsAb knockout cell pools, hyperosmolar media (506.0 ± 3.6 and $463.7.0 \pm 3.8$ mOsm/kg, respectively)

1 were prepared by adding 600 μ L of 5M NaCl to 30 mL of culture media. CHO-mAb and CHO-bsAb
2 knockout cell pools were seeded at 0.5×10^6 cells/mL in 125 mL Erlenmeyer flasks with 30 mL of the
3 hyperosmolar medium and incubated at 37 °C, 85 % humidity, 5 % CO₂, and 110 rpm. Cell viability
4 and density were measured daily. Culture supernatants were sampled daily and stored at -70 °C for
5 further analysis.

6

7 *2.13. Measurement of mAb and bsAb concentration*

8 mAb and bsAb concentrations were measured using enzyme-linked immunosorbent assay, as
9 previously described (Kim et al., 1998). The specific productivity was calculated from a plot of mAb
10 and bsAb concentrations against the time integral values of viable cell concentration (VCC), as
11 previously described (Renard et al., 1988).

12

13 *2.14. Quantitative real-time polymerase chain reaction (qRT-PCR)*

14 Total RNA was extracted using a Hybrid-R RNA extraction kit (GeneAll Biotechnology), and cDNA was
15 synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA),
16 according to the manufacturer's instructions. qRT-PCR was performed using iQ SYBR Green Supermix
17 (Bio-Rad) on a CFX96 Real-Time System (Bio-Rad), as previously described (Noh et al., 2018). The
18 relative expression levels were calculated using the $\Delta\Delta$ CT method and normalized to *Gapdh*. All
19 primer sequences used in this study are listed in Supplementary Table S2.

20

21 *2.15. Statistical analysis*

1 Values are represented as the mean \pm standard deviation. Data were analyzed using a two-tailed
2 Student's *t*-test, and the difference between the means was considered statistically significant at
3 $P < 0.05$.

4

5 **3. Results**

6 *3.1. Establishment of a virus-free, CHO-K1 genome-wide CRISPR knockout screening platform*

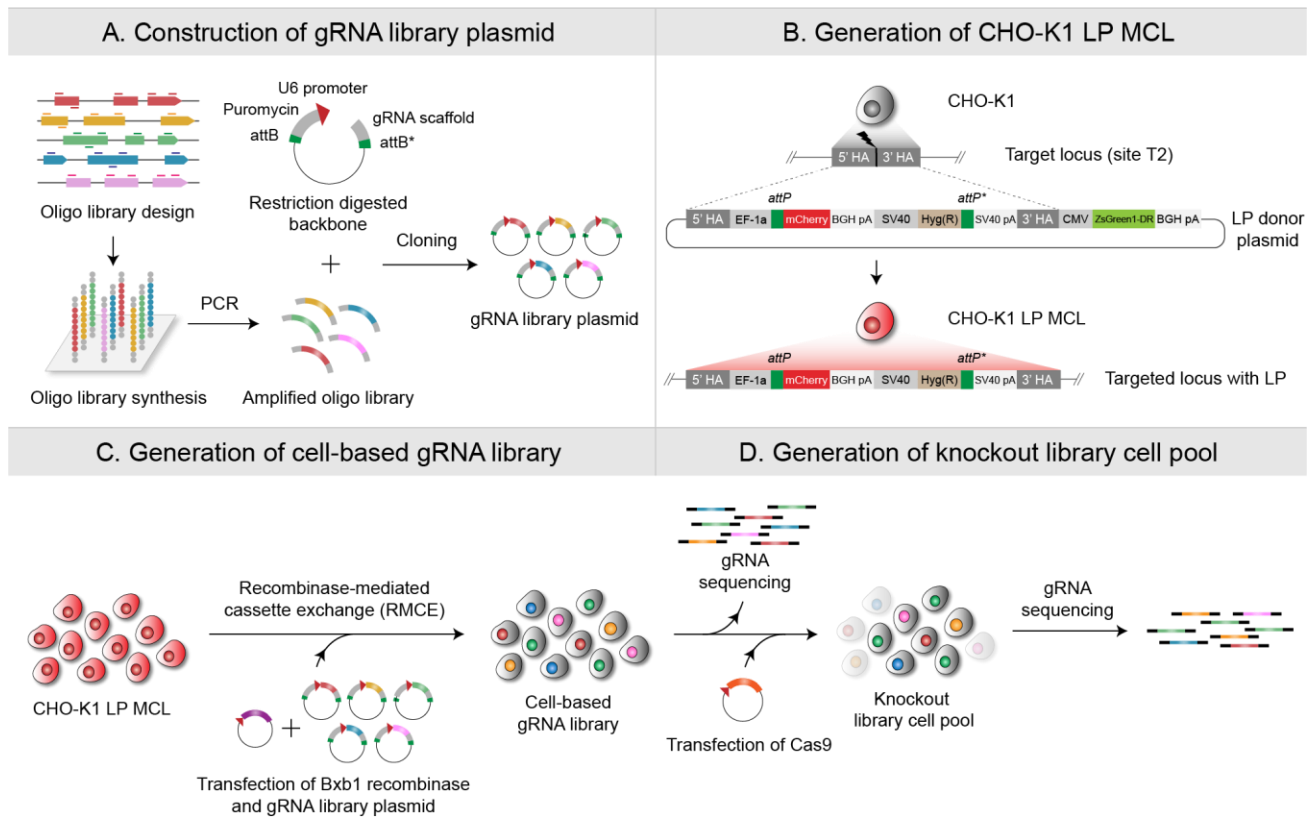
7 A schematic illustration of the virus-free RMCE-based CRISPR knockout library platform is shown in
8 Fig. 1. A CHO genome-wide CRISPR knockout library consisting of 111,651 gRNAs targeting 21,585
9 genes was designed, as described in the Materials and methods section (Supplementary Data File 1).
10 gRNAs were synthesized and cloned into an RMCE donor plasmid, harboring a gRNA expression
11 cassette and recombinase target sites. The cloned gRNA library plasmid was amplified and sequenced
12 using NGS to determine the gRNA distribution. The coverage of the gRNA library plasmid was 99.9%
13 and the skew ratio was 1.89, indicating sufficient representation with minimal bias. Next, to establish
14 an RMCE platform in CHO-K1 cells, a landing pad harboring the mCherry gene and recombinase target
15 sites was integrated into CHO-K1 cells. Based on homogeneous mCherry expression, clone #17 was
16 selected, which will be referred to as “MCL” hereafter (Supplementary Fig. S1). To improve the RMCE
17 efficiency of MCL, we generated an NLS-Bxb1 recombinase plasmid, which increased the efficiency
18 by 1.6-fold compared to that of the control Bxb1 recombinase plasmid (Supplementary Fig. S4).

19 To introduce a gRNA library into MCL using RMCE, MCL was transfected with a gRNA library and
20 NLS-Bxb1 recombinase plasmids, ensuring coverage of approximately 500 cells per gRNA. For
21 enrichment of cells harboring the integrated gRNA library, the RMCE cell pool was treated with 10

1 $\mu\text{g}/\text{mL}$ puromycin on day 3, and the puromycin selection was performed for 11 days (Fig. 2A and B).
2 RMCE-positive cells were enriched from 16.4 to 99.4%, as evidenced by the percentage of mCherry-
3 negative cells (Fig. 2C). To verify the representation of the gRNA library, gRNA sequences in the
4 genomic DNA from the cell-based gRNA library were amplified and sequenced using NGS. The
5 coverage of cell-based gRNA library was 99.7% and the skew ratio was 1.98, showing an even
6 distribution similar to the plasmid library (Fig. 2D and E). Thus, the gRNA library was sufficiently
7 represented in the cell-based gRNA library.

8 Knockout library cells were generated by the transient transfection of Cas9-BSD plasmid, followed
9 by three days of blasticidin selection to enrich the transfected cells (Fig. 3A). Eight days and sixteen
10 days after Cas9 transfection, the recovered cells were harvested for NGS analysis. To assess the
11 representation of the gRNA library, gRNA sequences in the genomic DNA from the knockout library
12 cell pools were amplified and sequenced using NGS. As expected, Cas9 transfection perturbed the
13 distribution of the gRNA library. The percentage of depleted gRNAs and genes gradually increased
14 after Cas9 transfection, as shown in Fig. 3B and C, indicating that the genes essential for cell survival
15 were affected. Sixteen days after Cas9 transfection, the generated knockout library cells were
16 screened.

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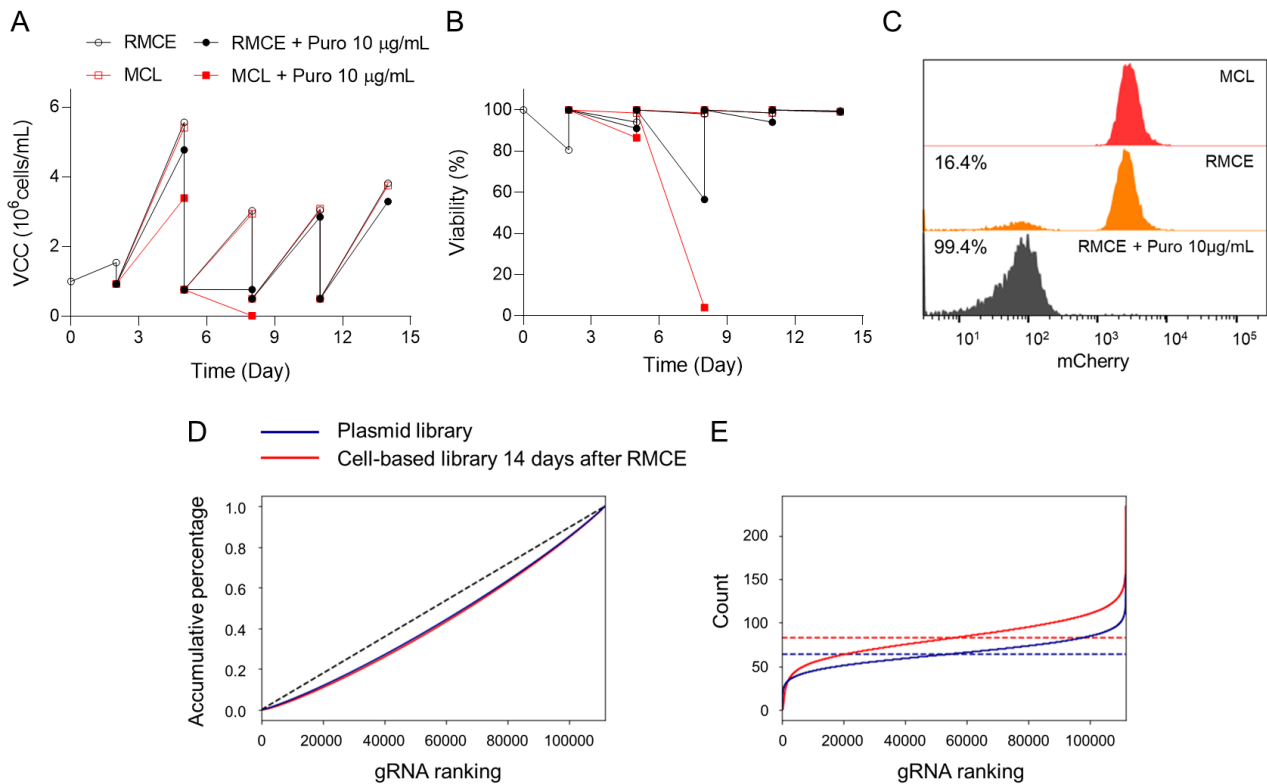
2 **Fig. 1. Establishment of a virus-free CHO-K1 genome-wide CRISPR knockout screening platform.** (A)
 3 CHO-K1 CRISPR knockout guide RNAs (gRNAs) were designed and cloned into a gRNA scaffold
 4 backbone plasmid containing the puromycin resistance gene flanked by attB and attB^{mut}
 5 recombination sites. (B) CHO-K1 landing pad (LP) master cell line (MCL) harboring mCherry and
 6 hygromycin resistance genes flanked by attP and attP^{mut} recombination sites was generated via
 7 CRISPR/Cas9-based targeted integration. (C) CHO-K1 LP MCL was transfected with Bxb1 recombinase
 8 and gRNA library plasmid to generate a cell-based gRNA library. (D) CHO knockout library cell pool
 9 was generated via Cas9 plasmid transfection. To verify the representation of gRNA library in cell-based
 10 gDNA library and knockout library cells, gRNA sequences were sequenced using next-generation
 11 sequencing (NGS).

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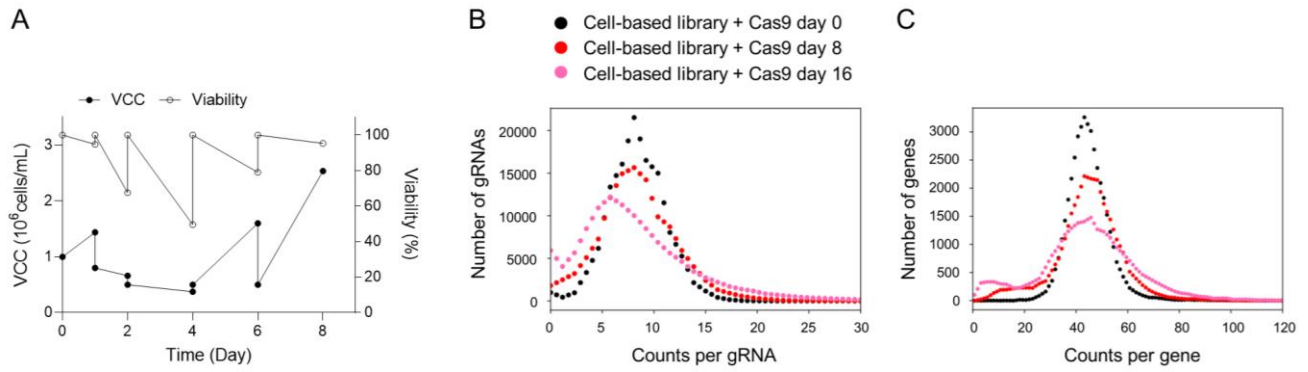
2 **Fig. 2. Generation of a cell-based gRNA library and representation of gRNA coverage.** Profiles of (A)
 3 cell growth (viable cell concentration, VCC) and (B) viability of RMCE cell pool (black empty circle),
 4 RMCE cell pool with 10 µg/mL of puromycin (black full circle), MCL (red empty square), and MCL with
 5 10 µg/mL of puromycin (red full square). On day 0, MCL was transfected with NLS-Bxb1 recombinase
 6 and gRNA library plasmids to obtain the RMCE cell pool. On day 2, the cells were sub-cultured. On
 7 day 3, RMCE cell pool and MCL control cells were treated with 10 µg/mL of puromycin for 11 days. (C)
 8 Flow cytometry analysis of the RMCE cell pool. Fourteen days after RMCE, cell populations expressing
 9 mCherry in MCL control (red), RMCE cell pool (orange), and RMCE cell pool with 10 µg/mL of
 10 puromycin (black) were measured. The percentage of mCherry-negative cells are shown. (D)
 11 Cumulative percentage of reads and (E) the number of read counts per gRNA in the plasmid library
 12 (blue solid line) and cell-based gRNA library 14 days after RMCE (red solid line). Dashed lines indicate
 13 the ideal models in the gRNA library.

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2 **Fig. 3. Generation of a knockout library cell pool and representation of library distribution.** (A) Cell
3 growth (viable cell concentration, VCC; full circle) and viability (empty circle) of the Cas9-transfected
4 knockout library cell pool. On day 0, puromycin enriched RMCE cell pool was transfected with Cas9-
5 BSD plasmid. On day 1, cells were treated with 10 $\mu\text{g}/\text{mL}$ of blasticidin for three days. Read
6 distributions of (B) gRNAs and (C) genes after Cas9 transfection in cell-based gRNA library. Cell based
7 library (black), eight days after Cas9 transfection (red), and 16 days after Cas9 transfection (magenta).

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1 3.2. Hyperosmotic stress screening

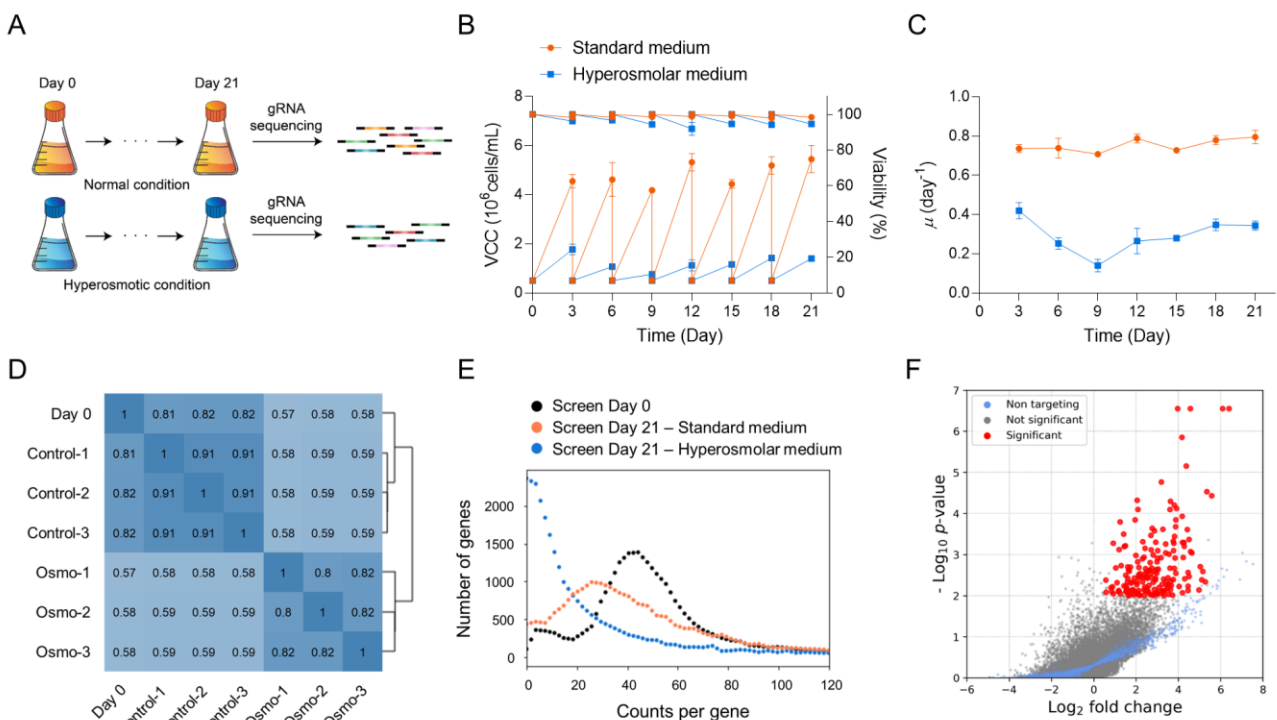
2 To determine the appropriate osmolality of the hyperosmolar medium for screening, knockout library
3 cells were cultured in a hyperosmolar medium in which osmolality increased linearly by 50 mOsm/kg
4 with NaCl addition (Supplementary Fig. S5A). Hyperosmolality negatively affected the cell growth and
5 viability (Supplementary Fig. S5B and C). Osmolality of the hyperosmolar medium for screening was
6 determined to be 460 mOsm/kg, which was the highest osmolality that the cells could tolerate
7 (Supplementary Fig. S5D).

8 Knockout library cells were seeded at 0.5×10^6 cells/mL in 1 L Erlenmeyer flasks containing 400 mL
9 of the standard or hyperosmolar medium and passaged every three days (Fig. 4A). Cell cultures were
10 performed in triplicate. Initially, cell growth was significantly suppressed in the hyperosmolar medium
11 than in the standard medium (Fig. 4B). On day 9, the specific growth rate (μ) of knockout library cells
12 in the standard medium was $0.71 \pm 0.01 \text{ day}^{-1}$, while that in the hyperosmolar medium was $0.14 \pm$
13 0.03 day^{-1} (Fig. 4C). However, the μ of cells in the hyperosmolar medium gradually increased and
14 became saturated on day 21, while that in the standard medium remained constant during the culture.
15 To evaluate the changes in gRNAs in standard and hyperosmolar media, cells were harvested on day
16 21 and subjected to NGS analysis.

17 To determine the gRNA abundance and distribution, computational analysis was conducted using
18 MAGeCK (Supplementary Data File 2). The gRNA read counts of cells sampled on day 21 were
19 compared to those of cells sampled on day 0. When correlated with cells sampled on day 0, cells
20 sampled on day 21 in the hyperosmolar medium (correlation coefficient of 0.58) showed less
21 correlation than cells sampled on day 21 in the standard medium (correlation coefficient of 0.82) (Fig.
22 4D). The first principal component obtained via principal component analysis showed clustering of

1 cells on days 0 and 21 in the standard medium, while it showed separation with 60% of variation of
 2 cells on days 0 and 21 in the hyperosmolar medium (Supplementary Fig. S6). The variation can be
 3 explained by the perturbation in the read distribution, as the percentage of depleted gRNA target
 4 genes significantly increased after 21 days of cultivation in the hyperosmolar medium (Fig. 4E). Next,
 5 the fold-change in gRNAs between normal and hyperosmotic conditions was evaluated. The α RRA
 6 algorithm was used to rank genes by combining fold-change data from all gRNAs, and 180 significantly
 7 enriched genes were found on the screen (Fig. 4F). Taken together, hyperosmotic stress (460
 8 mOsm/kg) provided sufficient selection pressure for the screen to generate perturbations in the cell
 9 pools.

10



11

12 **Fig. 4. Hyperosmotic stress screening.** (A) Schematic diagram illustrating hyperosmotic stress
 13 screening. Profiles of (B) cell growth (VCC), viability, and (C) μ in the standard medium (orange circle)
 14 and hyperosmolar medium (blue square). (D) Pearson correlation coefficient and sample hierarchical

1 clustering of the gRNA read counts between samples of day 0 and triplicates of the standard medium
2 (control) and hyperosmolar medium (osmo). (E) Read distribution of gRNA target genes. Day 0 (black),
3 average of triplicates of standard medium (orange), and average of triplicates of hyperosmolar
4 medium (blue). (F) Volcano plot showing the Log_2 fold-change and minus Log_{10} P -value for each gene.
5 Significantly enriched genes (P -value < 0.01) (red), non-targeting genes (blue), and not significant
6 genes (gray).

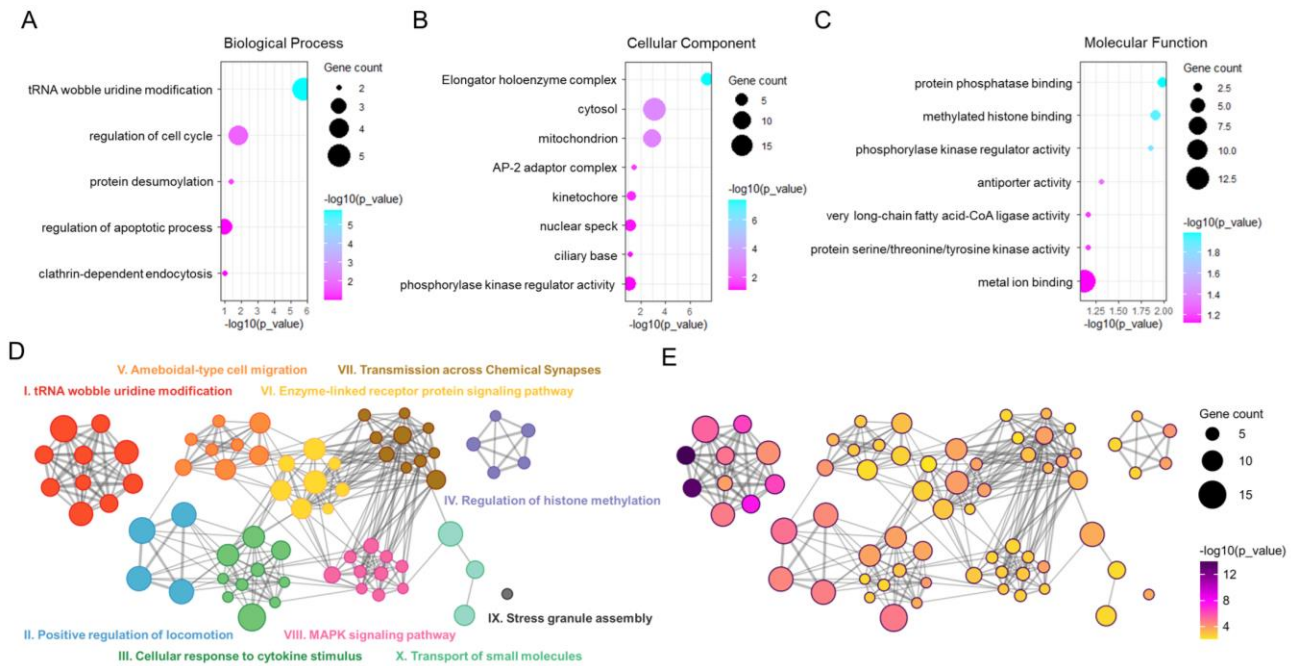
7

8 3.3. Functional enrichment analysis

9 To characterize the enriched genes on the screen, GO enrichment analysis was performed using
10 DAVID bioinformatics resources. GO enrichment analysis revealed the most significantly enriched GO
11 terms for tRNA wobble uridine modification in biological processes and elongator holoenzyme
12 complexes in cellular components (Fig. 5A and B). Both GO terms were composed of elongator
13 acetyltransferase complex subunit (*Elp*) genes, which are required for tRNA modifications. In addition,
14 biological processes of regulation of cell cycle, cellular components of the cytosol and mitochondria,
15 and molecular functions of protein phosphatase-binding and methylated histone-binding were
16 enriched (Fig. 5C).

17 To identify hyperosmotic stress-responsive pathways on a genome-wide scale, pathway and process
18 enrichment analyses were conducted using Metascape (Zhou et al., 2019). 10 functional clusters of
19 enriched biological terms were identified, and the relationships among the terms were visualized as
20 a network plot (Fig. 5D). A cluster related to tRNA wobble uridine modifications was significantly
21 enriched (Fig. 5E). Clusters related to the regulation of cell migration, cellular response to cytokine
22 stimulus, regulation of histone methylation, mitogen-activated protein kinase (MAPK) signaling
23 pathways, and transportation were also enriched. Raw data for pathway and process enrichment
24 analyses are summarized in Supplementary Data File 3.

1



2

3 **Fig. 5. Functional enrichment analysis using enriched genes to identify hyperosmotic stress-**
 4 **responsive gene networks.** Bubble plots showing enriched GO terms in (A) biological processes, (B)
 5 cellular components, and (C) molecular functions. The size of each circle indicates the number of
 6 genes that are enriched in the GO term. Network plots showing pathway and process enrichment
 7 analysis colored by (D) cluster and (E) *P*-value. Each node represents an individual enriched term. A
 8 connection between nodes represents Kappa similarity above 0.3, forming a network cluster. The
 9 node size indicates the number of genes enriched in the node.

10

11 **3.4. Validation of candidate genes in CHO-K1 cells**

12 To generate a robust data-set and prioritize genes from 180 significantly enriched genes derived from
 13 MAGeCK, computational analysis was conducted using another analysis tool, PinAPL-Py, and 58
 14 significantly enriched genes were identified (Supplementary Data File 4). Among the 58 genes, 52
 15 genes were enriched in both MAGeCK and PinAPL-Py analyses. To prioritize genes, the top 100 ranking
 16 genes were filtered using the MAGeCK aRRa score, and 32 genes were sorted (Fig. 6A). *Fastkd1* and

1 *Zfr* were the highest scoring genes, and all gRNAs targeting *Fastkd1* and *Zfr* showed significant
2 enrichment in the screen. GO enrichment analysis was conducted to characterize the functions of
3 these 32 genes. Three enriched GO terms of biological processes were found to be tRNA wobble
4 uridine modification, regulation of mRNA stability, and small molecule catabolic processes (Fig. 6B).
5 In addition to the three enriched GO terms, various gene functions, such as ion transport, regulation
6 of the cell cycle, endocytosis, and cell junctions were identified. Detailed functions of the 32 genes
7 are summarized in Table 1.

8 To verify the screening results, knockout cell pools for the 32 candidate genes were generated using
9 all-in-one CRISPR/Cas9 plasmids. The knockout efficiency of the all-in-one CRISPR/Cas9 system was
10 estimated by measuring the percentage of mCherry-negative cells in MCL using an mCherry-targeting
11 all-in-one CRISPR/Cas9 plasmid and increased to 97.0% via blasticidin selection (Supplementary Fig.
12 S7). A total of 32 knockout cell pools were cultured for 10 days in a 12-well plate containing the
13 hyperosmolar medium used for screening. VCCs were measured every other day. Cell cultures were
14 performed in three separate times.

15 Overall, 29 out of the 32 candidate gene knockout cell pools showed increased μ or maximum VCC
16 (MVCC) in the hyperosmolar medium (Fig. 6C and D). In addition, eight of the 29 candidate gene
17 knockout cell pools showed a significant increase in both μ and MVCC. (Fig. 6C and D). Interestingly,
18 five (*Fastkd1*, *Zfr*, *Acsf3*, *Elp4*, *Elp5*) of the eight candidate genes were the most highly enriched genes
19 in the screen.

20 *Kti12* knockout pool showed the highest time integral of VCC (IVCC) of $23.6 \pm 1.4 \times 10^6$ cells/mL-day,
21 which is 1.6-fold higher than that of the NT control pool (Fig. 6E). In addition, the majority of
22 candidate gene knockout pools showed higher viability than the NT control pool on day 10

1 (Supplementary Fig. S8). *Elp4* knockout pool showed the highest viability of 80% on day 10, whereas
 2 the viability of the NT control pool was 42% on day 10. Thus, knockout validation narrowed the
 3 candidate genes from the 32 candidate genes screened using hyperosmotic stress screening. Seven
 4 genes, whose knockout showed the highest increase in both MVCC and IVCC, were further validated.

5

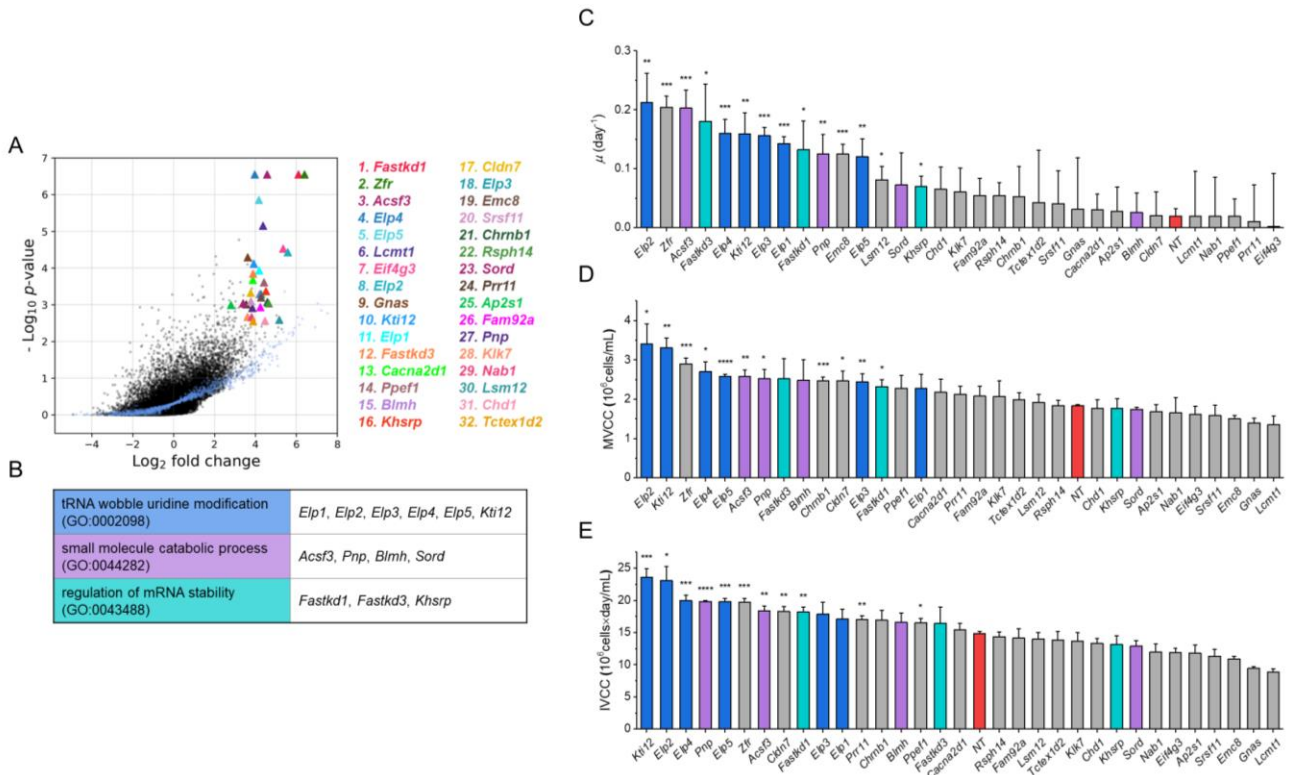
6 **Table 1.** List of 32 candidate genes significantly enriched in the analysis.^a

Ranking	Gene symbol	Gene description	Function
1	<i>Fastkd1</i>	FAST kinase domains 1	mitochondrial RNA processing
2	<i>Zfr</i>	zinc finger RNA binding protein	nucleic acid binding
3	<i>Acsf3</i>	acyl-CoA synthetase family member 3	fatty acid metabolic process
4	<i>Elp4</i>	elongator acetyltransferase complex subunit 4	tRNA wobble uridine modification
5	<i>Elp5</i>	elongator acetyltransferase complex subunit 5	tRNA wobble uridine modification
6	<i>Lcmt1</i>	leucine carboxyl methyltransferase 1	protein methylation
7	<i>Eif4g3</i>	eukaryotic translation initiation factor 4 gamma 3	translation initiation
8	<i>Elp2</i>	elongator acetyltransferase complex subunit 2	tRNA wobble uridine modification
9	<i>Gnas</i>	GNAS complex locus	receptor signaling
10	<i>Kti12</i>	KTI12 chromatin associated homolog	tRNA wobble uridine modification
11	<i>Elp1</i>	elongator acetyltransferase complex subunit 1	tRNA wobble uridine modification
12	<i>Fastkd3</i>	FAST kinase domains 3	mitochondrial RNA processing
13	<i>Cacna2d1</i>	calcium voltage-gated channel auxiliary subunit alpha2delta 1	calcium ion transport
14	<i>Ppef1</i>	protein phosphatase with EF-hand domain 1	calcium ion binding
15	<i>Blmh</i>	bleomycin hydrolase	small molecule catabolic process
16	<i>Khsrp</i>	KH-type splicing regulatory protein	mRNA processing
17	<i>Cldn7</i>	claudin 7	cell junction
18	<i>Elp3</i>	elongator acetyltransferase complex	tRNA wobble uridine

		subunit 3	modification
19	<i>Emc8</i>	ER membrane protein complex subunit 8	ER membrane
20	<i>Srsf11</i>	serine and arginine rich splicing factor 11	mRNA processing
21	<i>Chrnβ1</i>	cholinergic receptor nicotinic beta 1 subunit	cation transport
22	<i>Rsph14</i>	radial spoke head 14 homolog	Unknown
23	<i>Sord</i>	sorbitol dehydrogenase	small molecule catabolic process
24	<i>Prr11</i>	proline rich 11	regulation of cell cycle
25	<i>Ap2s1</i>	adaptor related protein complex 2 subunit sigma 1	regulation of endocytosis
26	<i>Fam92a</i>	CBY1 interacting BAR domain containing 1	cell junction
27	<i>Pnp</i>	purine nucleoside phosphorylase	small molecule catabolic process
28	<i>Klk7</i>	kallikrein related peptidase 7	response to stimulus
29	<i>Nab1</i>	NGFI-A binding protein 1	regulation of transcription
30	<i>Lsm12</i>	LSM12 homolog	Unknown
31	<i>Chd1</i>	chromodomain helicase DNA binding protein 1	chromatin remodeling
32	<i>Tctex1d2</i>	dynein light chain Tctex-type 2B	cilium assembly

1 ^a Analysis was performed using Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout
2 (MAGeCK) and Platform-independent Analysis of Pooled screens using Python (PinAPL-Py).

3



1

2 **Fig. 6. Validation of the candidate genes.** (A) Log₂ fold-change and minus Log₁₀ *P*-value in MAGeCK
 3 analysis for the top-scoring 32 candidate genes. The number in front of the gene name indicates its
 4 ranking in the analysis. Genes are indicated by triangles with labeled color. Non-targeting genes are
 5 indicated by blue dots and not significant genes are indicated by black dots. (B) Enriched GO terms
 6 and genes. (C) μ , (D) MVCC, and (E) IVCC of the 32 candidate gene knockout pools. μ was calculated
 7 based on the values of VCC during the exponential phase (days 2-6). Columns in blue represent genes
 8 in the GO term of tRNA wobble uridine modification, in purple represent genes in the GO term of
 9 small molecule catabolic process, in cyan represent genes in the GO term of regulation of mRNA
 10 stability, and in red represent the NT control. Asterisks (*) indicate the significant difference compared
 11 to the NT control. Error bars in the plot represent the standard deviations of three biological replicates.
 12 An unpaired two-tailed *t*-test was used to determine the significance of the mean difference. **P* <
 13 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

14

15

16

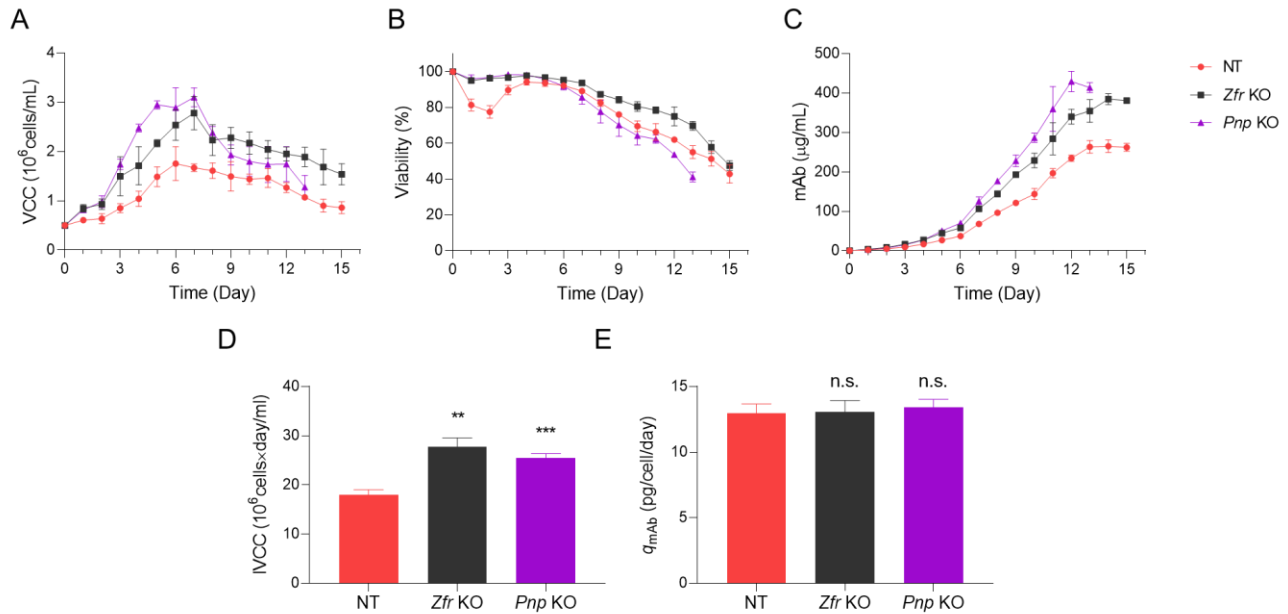
17 *3.5. Assessment of gene knockout in mAb-producing rCHO cell lines*

1 To further investigate the effects of target gene knockout in mAb-producing rCHO cell lines, knockout
2 cell pools for seven candidate genes (*Zfr*, *Acsf3*, *Elp2*, *Elp4*, *Elp5*, *Kti12*, and *Pnp*) were generated using
3 all-in-one CRISPR/Cas9 plasmids. Knockout of each target gene was verified via mRNA expression
4 analysis, which showed decreased mRNA expression levels for each target gene (Supplementary Fig.
5 S9). NT control and seven candidate gene knockout pools were cultured in the hyperosmolar medium,
6 and VCCs and mAb concentrations were measured on day 6. Among the seven candidate gene
7 knockout pools, *Zfr* and *Pnp* knockout pools showed significant increase in both cell growth and mAb
8 productivity (Supplementary Fig. S10). To assess the growth profiles of *Zfr* and *Pnp* knockout pools in
9 the hyperosmolar medium, NT control and *Zfr* and *Pnp* knockout cell pools were cultured in 125 mL
10 Erlenmeyer flasks with 30 mL of hyperosmolar medium (506.0 ± 3.6 mOsm/kg). VCC was measured
11 daily. Experiments were performed in three separate times.

12 Compared to the NT control, *Zfr* and *Pnp* knockout cell pools showed a higher VCC during culture
13 (Fig. 7A). The viability of NT control cell pools dropped to 77.5 ± 3.5 % on day 2, while that of *Zfr* and
14 *Pnp* knockout pools remained above 95% (Fig. 7B). IVCCs of *Zfr* knockout ($27.8 \pm 1.8 \times 10^6$ cells/mL-day)
15 and *Pnp* knockout ($25.4 \pm 0.9 \times 10^6$ cells/mL-day) cells were 1.5 and 1.4-fold higher than that of NT
16 ($18.0 \pm 1.1 \times 10^6$ cells/mL-day) cells, which resulted in increased mAb production (Fig. 7C and D). The
17 maximum mAb concentrations of *Pnp* knockout (429.4 ± 25.8 mg/L) and *Zfr* knockout (384.7 ± 13.8
18 mg/L) cells were 1.6- and 1.5-fold higher than that of NT (265.1 ± 15.8 mg/L) cells (Fig. 7C). However,
19 the specific productivities (q_{mAb}) of the knockout cell pools were not significantly different from that
20 of the NT control cells (Fig. 7E). *Zfr* and *Pnp* knockout pools were also cultured in the standard medium,
21 but no significant differences were observed compared to the NT control cells (data not shown).
22 Therefore, knockout of *Zfr* and *Pnp* conferred osmotic stress resistance under hyperosmotic

1 conditions and increased the cell growth and mAb production in CHO cells.

2



3

4 **Fig. 7. Batch cultures of *Zfr* and *Pnp* knockout CHO-mAb pools.** Profiles of (A) cell growth (VCC), (B)
5 viability, (C) mAb concentration, (D) IVCC, and (E) q_{mAb} of *Zfr* and *Pnp* knockout CHO-mAb pools and
6 NT control pool in the hyperosmolar medium. Asterisks (*) indicate the significant difference
7 compared to the NT control. Error bars in the plot represent the standard deviations of three
8 biological replicates. An unpaired two-tailed *t*-test was used to determine the significance of the
9 mean difference. n.s. $P > 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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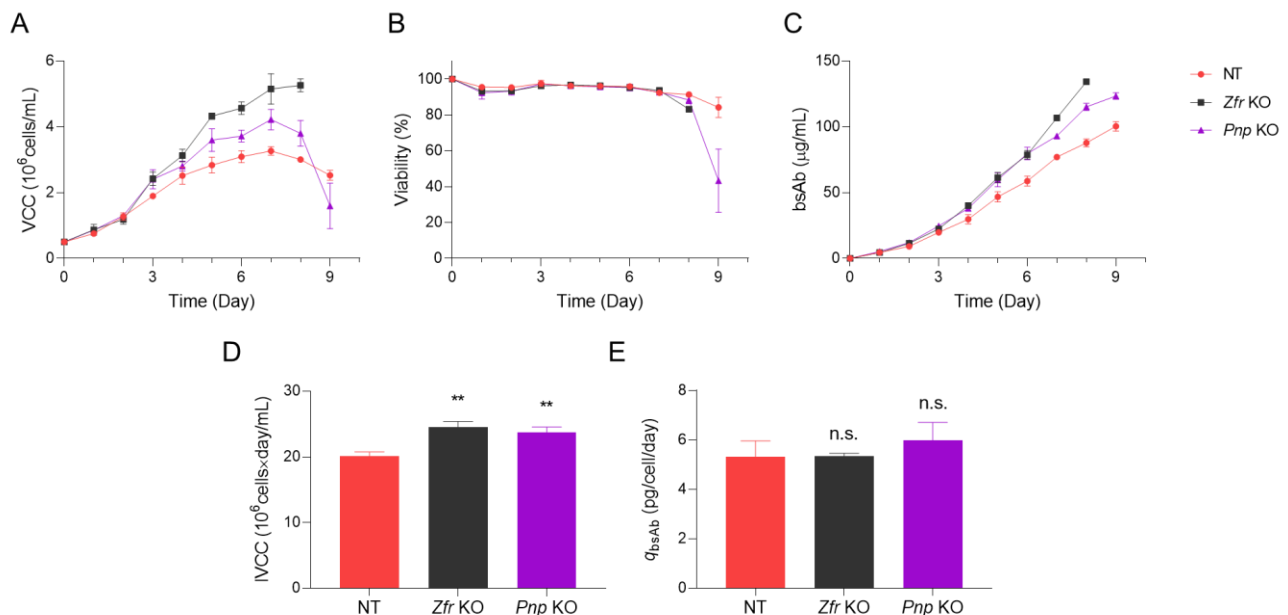
11 3.6. Assessment of gene knockout in bsAb-producing CHO-S cell lines

12 To determine whether knockout of *Zfr* and *Pnp* confer osmotic stress resistance in rCHO cell lines
13 producing different antibody products generated from different lineages, CHO-S cell lines producing
14 bsAbs were transfected with the NT control and *Zfr* and *Pnp* targeting all-in-one CRISPR/Cas9 plasmids.
15 The relative mRNA expression levels of *Zfr* and *Pnp* in *Zfr* and *Pnp* knockout cell pools, respectively,
16 showed successful gene knockout of each target gene (Supplementary Fig. S11). Knockout cell pools

1 were cultured in 125 mL flasks with 30 mL of hyperosmolar medium (463.7 ± 3.8 mOsm/kg). VCC was
2 measured daily. Experiments were performed in three separate times.

3 *Zfr* and *Pnp* knockout cell pools showed a higher VCC than the NT control cells during culture (Fig.
4 8A). IVCCs of *Zfr* knockout ($24.5 \pm 0.9 \times 10^6$ cells/mL·day) and *Pnp* knockout ($23.8 \pm 0.8 \times 10^6$
5 cells/mL·day) cells were 1.2-fold higher than that of NT ($20.1 \pm 0.6 \times 10^6$ cells/mL·day) cells (Fig. 8D).
6 The maximum bsAb concentrations of *Zfr* knockout (134.3 ± 1.6 mg/L) and *Pnp* knockout (123.4 ± 2.6
7 mg/L) cells were 1.3- and 1.2-fold higher than that of NT (100.4 ± 3.6 mg/L) cells (Fig. 8C). However,
8 the q_{mAb} of knockout cell pools was not significantly different from that of the NT control cells (Fig.
9 8E). Therefore, knockout of *Zfr* and *Pnp* conferred osmotic stress resistance and increased the cell
10 growth and bsAb production in CHO cells.

11



12

13 **Fig. 8. Batch cultures of *Zfr* and *Pnp* knockout CHO-bsAb pools.** Profiles of (A) cell growth (VCC), (B)
14 viability, (C) mAb concentration, (D) IVCC, and (E) q_{mAb} of *Zfr* and *Pnp* knockout CHO-bsAb pools and
15 NT control pool in the hyperosmolar medium. Asterisks (*) indicate the significant difference

1 compared to the NT control. Error bars in the plot represent the standard deviations of three
2 biological replicates. An unpaired two-tailed t -test was used to determine the significance of the
3 mean difference. n.s. $P > 0.05$, and $**P < 0.01$.

4

5 **4. Discussion**

6 Being powerful mammalian expression platforms for the production of therapeutic proteins,
7 including mAbs, CHO cells have been engineered to achieve high productivity and quality of
8 therapeutic proteins through knowledge-based approaches. However, empirical considerations for
9 finding novel targets for desired attributes have marginal effects, necessitating the development of
10 new strategies. Advances in CRISPR/Cas9 technology have generated a wealth of tools for genome-
11 scale approaches, such as genetic screening, which enables unbiased dissection of genes with
12 relevant phenotypes. Here, genome-wide CRISPR/Cas9 screening was performed in CHO cells to
13 identify novel targets associated with industrially relevant hyperosmotic stress conditions.

14 Most of the current experimental designs in CRISPR/Cas9 screening involve lentiviral transduction
15 of the gRNA library into Cas9 expressing cells, however, this can result in spurious gene editing of Cas9
16 guided by multiple gRNAs (Xiong et al., 2021). In addition, Cas9 expression can result in nuclease-
17 induced cellular toxicity due to the DNA damage response (Morgens et al., 2017; Tycko et al., 2019).
18 To circumvent these issues, RMCE-based gRNA integration followed by a transient Cas9 expression
19 platform was previously established, demonstrating high efficiency both in single integration of gRNA
20 and in Cas9 editing (Xiong et al., 2021). Using this platform, even distribution of gRNAs with high
21 coverage was accomplished in our cell-based library (Fig. 2D), and knockout library cells were
22 subsequently generated (Fig. 3).

23 Pooled screening requires a suitable selection pressure that can lead to perturbation of cell

1 proliferation or viability, such that enriched or depleted mutants can be effectively discriminated.
2 Osmotic tolerance differs among cell lines (Ryu et al., 2001). Thus, the appropriate hyperosmolar
3 medium for each rCHO cell line was determined throughout the osmolality test (Supplementary Fig.
4 S5; Fig. S12). Consequently, the hyperosmolar medium used for screening sufficiently generated
5 perturbations in the cell pools and elicited significantly enriched and depleted genes (Fig. 4).

6 Hyperosmotic stress inhibits cell proliferation and induces apoptosis by activating signaling
7 pathways mediated by MAPKs, including p38 and c-Jun N-terminal kinase (Zhou et al., 2016). As
8 expected, functional enrichment analysis of genes enriched in the screen identified enriched terms
9 of cell cycle arrest and the MAPK signaling pathway (Fig. 5). Representatively, *Pten*, *Nf2*, *Mapk14*, and
10 *Traf3* have been identified. These genes are known to inhibit cell proliferation, and their knockout
11 promotes cell proliferation (Brandmaier et al., 2017; Gurusamy et al., 2020; Xiao et al., 2005; Zhou et
12 al., 2021). Additionally, hyperosmotic stress induces cell volumetric changes that accompany various
13 alterations in transporters across the membrane (Hoffmann et al., 2009). We also found enriched
14 terms of transporters, which included genes, such as chloride voltage-gated channel (*Clcn4*), calcium-
15 activated cation channel (*Trpm4*), and solute carrier gene family. In particular, *Trpm4* can generate
16 osmotic gradients, and knockdown of *Trpm4* gene attenuates cell swelling, preventing negative
17 consequences in astrocytes (Stokum et al., 2018). Interestingly, while most functional terms were
18 connected to form a network, the term tRNA wobble modification was separated but showed the
19 most significant enrichment in functional analyses. Upon stress conditions, cells have been shown to
20 support efficient translation of stress-responsive genes favoring rare tRNAs with wobble-pairing by
21 altering tRNA abundance and modification (Frenkel-Morgenstern et al., 2012; Torrent et al., 2018),
22 and the alteration of tRNA modifications contributes to a translational regulatory mechanism in stress

1 response (Huang and Hopper, 2016).

2 Computational analyses identified 32 top-scoring genes, and the majority of knockout cell pools for
3 each candidate gene showed hyperosmotic stress-resistance in CHO-K1 cells, validating the screening
4 result (Fig. 6). Notably, knockout of *Fastkd1* and *Zfr*, which were the highest-scoring genes with
5 significant enrichment of all target gRNAs, significantly increased the cell growth under hyperosmotic
6 stress in CHO-K1 cells. In addition, perturbations of genes related to tRNA wobble modification (*Elp1*,
7 *Elp2*, *Elp3*, *Elp4*, *Elp5*, and *Kti12*), which showed significant enrichment in functional analysis, resulted
8 in high μ and MVCCs in CHO-K1 cells. While the hyperosmotic stress-resistant effect of knockout of
9 *Zfr* and *Pnp* persisted in CHO-mAb cell lines, consistent phenotypes were not reproduced for some
10 genes related to tRNA wobble modification (Supplementary Fig. S10). This can be explained by the
11 dynamics of tRNA pools and variations of stress-responsive genes in various cell types, thereby
12 causing to variations in cell lines (Gingold et al., 2012).

13 Hyperosmolality can increase q_p in CHO cells to different extents in different cell lines (Ryu et al.,
14 2000); therefore, we investigated the effects of gene perturbations upon hyperosmotic stress in CHO
15 cells of different origins, producing different products. Notably, perturbations of *Zfr* and *Pnp* genes in
16 CHO-mAb and CHO-bsAb cell lines also increased the cell growth under hyperosmotic culture
17 conditions and enhanced the product titers. However, this improvement was not attained by a
18 substantial increase in q_p (Fig. 7E; Fig. 8E). Because hyperosmotic stress leads to an increase in q_p
19 concomitant with cell cycle arrest, the enhancement of q_p may also be diminished by mitigating
20 growth inhibition. PNP is an enzyme that converts inosine to hypoxanthine in the purine degradation
21 pathway, and inosine enhances the cell proliferation of highly proliferating cells (Soares et al., 2015;
22 Yin et al., 2018). Therefore, we speculate that blockage of inosine conversion by the knockout of *Pnp*

1 enhanced the cell proliferation in this study. ZFR is a DNA- and RNA-binding protein; however, its
2 biological functions remain unknown. Hence, further investigations of the roles of ZFR should be
3 performed in future studies.

4 In conclusion, genome-wide CRISPR/Cas9 screening was performed in CHO-K1 cells, and novel genes
5 and functional clusters associated with hyperosmotic stress were identified. Knockout of target genes
6 increased the cell growth under hyperosmotic culture conditions, thereby enhancing the productivity.
7 Our findings demonstrate the beneficial values of the screening platform in providing novel insights
8 on hyperosmotic stress and identification of novel targets for rational cell engineering on a genome-
9 wide scale.

10

11 **Author statement**

12 **Su Hyun Kim:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft,
13 Writing – review & editing; **Seunghyeon Shin:** Validation, Investigation, Writing – review & editing;
14 **Minhye Baek:** Validation, Investigation, Writing – review & editing; **Kai Xiong:** Methodology, Software,
15 Validation, Resources; **Karen Julie la Cour Karottki:** Software, Formal analysis, Resources; **Hooman**
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19 Validation, Project administration, Funding acquisition, Writing – review & editing; **Gyun Min Lee:**
20 Conceptualization, Methodology, Validation, Project administration, Funding acquisition, Supervision,
21 Writing – review & editing

1

2 **Declaration of competing interest**

3 The authors declare no financial or commercial conflict interest.

4

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10

11 **Appendix A. Supplementary data**

12 Supplementary data to this article can be found online at

13

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