An arrayed CRISPR screen reveals Myc depletion to increase productivity of difficult-to-express complex antibodies in CHO cells

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Abstract

Complex therapeutic antibody formats, such as bispecifics (bsAbs) or cytokine fusions, may provide new treatment options in diverse disease areas. However, the manufacturing yield of these complex antibody formats in Chinese Hamster Ovary (CHO) cells is lower than monoclonal antibodies due to challenges in expression levels and potential formation of side products. To overcome these limitations, we performed a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)-based knockout (KO) arrayed screening of 187 target genes in two CHO clones expressing two different complex antibody formats in a production-mimicking set-up. Our findings revealed that Myc depletion drastically increased product expression (>40%) by enhancing cell-specific productivity. The Myc-depleted cells displayed decreased cell densities together with substantially higher product titers in industrially-relevant bioprocesses using ambr15 and ambr250 bioreactors. Similar effects were observed across multiple different clones, each expressing a distinct complex antibody format. Our findings reinforce the mutually exclusive relationship between growth and production phenotypes and provide a targeted cell engineering approach to impact productivity without impairing product quality. We anticipate that CRISPR/Cas9-based CHO host cell engineering will transform our ability to increase manufacturing yield of high-value complex biotherapeutics.

Key words: CHO; CRISPR/Cas9; arrayed KO screening; antibody production

Graphical Abstract

1. Introduction

The modular architecture of antibodies has been widely exploited to engineer a multitude of complex bispecific antibodies. This includes the number of antigen-binding sites, spatial relationship between different binding sites, molecule composition, size, flexibility, valency for each antigen and half-life (1). Bispecific heterodimeric antibody formats have been shown to extend the therapeutic target space with novel mechanisms of action through their dual specificity, e.g. delivery through biological barriers, effector cell recruitment or inactivation of receptors and ligands (2). To address these diverse mechanisms of action, bispecific antibodies (bsAbs) can be rationally designed in many formats,
ranging from two small antigen-binding fragments to large multidomain Immunoglobulin G (IgG)-like molecules. As of now, over 100 bsAbs are in clinical development with more than 30 different bsAb formats (3). This variety of bsAbs in clinical trials demonstrates that the field is utilizing a diverse panel of formats to fit the respective target product profile. Importantly, it has been recognized that the molecule format defines the functionality of the bsAbs (4). Identifying the most suitable format for a given mode of action requires novel in vitro assays during development, which enable screening of a large number of complex antibody formats for different antibody properties (5).

While the diversity of bsAb formats serves as a prerequisite to their extended therapeutic potential, the increasing complexity impairs a streamlined and reproducible production process. Their heterodimeric composition (3–4 different chains in contrast to two in IgG) poses inherent chain association problems, as 10–16 different antibody species can be formed with only one having the desired functionality (6). bsAb formats, therefore, require extensive optimization of the expression system with a focus on reducing the levels of undesired antibody species, besides yield and stability. Aiming to maximize the expression of individual chains of a bsAb is inadequate for reducing undesired antibody species, rather optimal subunit gene dosage of each chain is required to prevent the formation of unwanted product species (7). Specific molecule design strategies, e.g. knobs-into-holes technology combined with CrossMab technology greatly enhance correct chain association (8, 9). Additional progress was achieved by excluding position-specific effects by using targeted integration (TI) host(s), allowing for homogeneous gene expression via integrating predefined expression vectors in the TI landing pad (10, 11). Regardless, the manufacturing yields of complex antibody formats are lower than monoclonal antibodies, making them ‘difficult-to-express’ (DTE).

CHO cells serve as the preferred expression system for the commercial production of therapeutic proteins (12). During the development of stable recombinant CHO cell lines, high-producing clones are generated and tested extensively to identify lead clone(s) for commercial manufacturing use. Notably, clone performance early during the cell line development process and at small scales (shake flask, multiwells) is not necessarily predictive of industrial scale performance (13). Constant changes in CHO genome via rearrangements and DNA methylation patterns result in large diversity of genotypes spontaneously arising in CHO cell populations (14). This instability is a constant and unpredictable feature with no population being stable or uniform over an extended period of time (15, 16). However, the success of CHO cells in biomanufacturing is at least partly based on this flexibility to adapt to any given environment quickly (17). Importantly, once cells have physiologically adapted to their new environment, i.e. the bioreactor set-up, their phenotype remains stable, as long as this environment is kept (17). The field is, therefore, relying on miniaturized microbioreactor (MBR) systems as downscale models to closely mimic the production process of current good manufacturing practice in large-scale bioreactors (18). The MBR systems have been shown to reproduce the performance of CHO clones under conventional bioreactor production conditions (with regulated pH, dissolved oxygen, CO₂ and nutrition levels) (19).

The increasing demand of high-value DTE molecules is illustrated by more than 100 bsAbs currently in clinical trials, which underlines the need for developing high-yield expression systems. Clustered regularly interspaced short palindromic repeats (CRISPR) technology is a promising method to engineer multiple cellular features for improving the CHO expression system. Targeted engineering of host cells by CRISPR/Cas9 has already been successfully utilized to improve cellular characteristics in downscale model systems, e.g. host cell protein contamination (20), improved productivity (21, 22) and product glycosylation patterns (23).

Typically, due to low single-gene knockout (KO) efficiency at the pool level, this involves isolation of KO subclones to study their individual phenotypes. However, KO subclones can show remarkable phenotypical heterogeneity (24). Consequently, it remains unclear whether any observed effect reflects a general phenotype of the introduced KO or is solely a feature of a particular clone (25, 26).

Here, we devised a pooled single-gene-KO-based approach to determine the average effect of any genetic perturbation in order to obtain more reliable screening results. We screened the depletion of 187 target genes for their effect on the expression of two different DTE antibody formats in two CHO clones. While several hits increased titer >10%, we demonstrate that Myc KO strongly increased product expression (>40%) by enhancing cell-specific productivity under bioreactor conditions. Myc KO cells displayed decreased cell densities together with substantially higher product titers in industrial-relevant bioprocesses using ambr15 and ambr250 bioreactors. These effects were consistent across multiple additional CHO clones each expressing a distinct complex antibody format.

2. Materials and Methods

2.1 Cell culture

All cell lines were created using a previously generated CHO Host Cell Line (International patent publication number WO 2019/126634 A2). CHO cells were cultured in a proprietary Dulbecco’s Modified Eagle’s Medium/F12-based medium in 125–500-ml shake flask vessels at 150 rpm, 37°C, 80% RH and 5% CO₂. Cells were passaged at a seeding density of 3–6 x 10⁶ cells/ml every 3–4 days. Pools of cells that stably express bsAb molecules were generated as described (10). Briefly, expression plasmids were transfected into CHO cells by MaxCyte STX electroporation (MaxCyte, Inc.). The transfected cells were then selected and the expression of mAb was confirmed by flow cytometry via human IgG staining (BD FACs Canto II flow cytometer, BD). CHO clones were selected after single cell cloning by limited dilution, titer and binder validation by enzyme-linked immunosorbent assay and evaluation of volumetric productivity in fed-batch production assays in ambr15 and ambr250 bioreactors (Sartorius AG).

2.2 Fed-batch production assay

Fed-batch production cultures were performed in shake flasks or ambr15 or ambr250 bioreactors (Sartorius AG) with proprietary chemically-defined production media. Cells were seeded as indicated between 2 and 15 x 10⁶ cells/ml on Day 0 of the production stage after adaptation to production media during two passages. Cultures received daily proprietary feed medium after Day 3 and additional feed bolus on Days 3 and 7, with optional bolus at Day 10. Cells were cultivated for 14 days. Production in the ambr15 system was operated at set points of 36°C, DO 30%, pH 7.0 and an agitation rate of 1200 rpm. Production in the ambr250 system was operated at set points of 35°C, DO 30%, pH 7.0 and an agitation rate of 1300 rpm with shift to 1600 rpm on Day 3.5.

2.3 Offline sample analysis

Process parameters were analyzed with Osmomat Auto (Gonotec GmbH, Berlin, Germany) for the measurement of osmolality and a
Cedex Bio HT Analyzer (Roche Diagnostics GmbH) for the measurement of product and selected metabolite concentrations. Total cell count, viable cell concentration and average cell diameter were measured by Cedex HiRes Analyzer (Roche Diagnostics GmbH).

Integrated cell volume and specific productivity rates (SPRs) for each condition were calculated based on the relationship shown:

\[
IVCV_n = IVCV_{n-1} + \frac{VCV_n + VCV_{n-1}}{2} \cdot (t_n - t_{n-1})
\]

\[
SPR_{deltaCV} = \frac{dcPRD}{dIVCV} = \frac{cPRD_n - cPRD_{n-1}}{IVCV_n - IVCV_{n-1}} \cdot 24
\]

2.4 Generation of pooled single-gene KOs and KO confirmation

Targets were deliberately chosen and guides were designed using the Geneious Prime software (version 2020.2.4, Off-target Library: CHO Reference Genome GCA_003668045.1_CriGri-PICR_genomic). All single guide ribonucleic acid (sgRNA) sequences and verification primers are listed in Supplementary Table S1. For KO introduction, CHO producer clones were transfected with ribonucleoprotein (RNP) complexes comprising gene-targeting sgRNA and Cas9 protein using the MaxCyte STX electroporation system (MaxCyte, Inc.). Guide RNA-Cas9 RNP complexes were prepared by mixing sgRNA (total of 40 pmol for each nucleofection) with an equimolar amount of Cas9 protein (TrueCut Cas9 Protein v2, Thermo Fisher Scientific Inc.) followed by incubation at room temperature (RT) for 20 min. Cells were washed in phosphate-buffered saline (PBS) (300 g, 5 min) and resuspended in the respective electroporation buffer (MaxCyte EP Buffer, Maxcyte Inc.).

Genomic DNA was extracted using QuickExtract™ DNA Extraction Solution (Lucigen) according to the manufacturer’s instructions after cells have recovered from transfection (6-8 days post transfection). Polymerase chain reaction (PCR) (98°C for 30 s; 35 times: 98°C for 5 s, 60°C for 20 s and 72°C for 90 s/72°C for 90 s) was performed with the Q5 Polymerase 2× Master Mix (New England Biolabs, Inc.). Amplicons were purified with QIAquick® 96 PCR Purification Kit (Qiagen). Sanger sequencing was performed by BIOSCRIP (Belgach, Switzerland). The PCR products produced were analyzed via electrophoresis on 2% agarose.

KO scores were assessed by the offline version of the Inference of CRISPR Edits software for analyzing Sanger sequencing data (available at https://github.com/synthegeo-open/ice).

2.5 Arrayed CRISPR KO screening workflow

For KO introduction, two CHO clones producing Molecules 1 and 2 (Figure 2a) were transfected with RNP complexes comprising gene-targeting sgRNA and Cas9 protein using the Lonza 96-well shuttle system (Lonza Group Ltd). Guide RNA-Cas9 RNP complexes were prepared by mixing sgRNA (40 pmol/sgRNA) with an equimolar amount of Cas9 protein (TrueCut Cas9 Protein v2, Thermo Fisher Scientific Inc.) followed by incubation at RT for 20 min. Electroporation (program DS 167) was performed after cells were washed in PBS (300 g, 5 min) and resuspended in the respective electroporation buffer (SF Cell Line 4D-Nucleofector™ X Kit, Lonza Group Ltd.). After KO introduction, cells were cultivated in 24-well plates (Thermo Fisher Scientific Inc.) for 5 days. Genomic DNA for KO confirmation was extracted on Day 7 as described in the previous section. Cells were subsequently cultivated and, therefore, transferred in 24 deep-well plates (Porvair Sciences Ltd). Batch production cultures were performed in 24 deep-well plates with proprietary chemically-defined production media after upscale to a total volume of 4.5 ml. Cells were seeded with 5 × 10^6 cells/ml on Day 0 of the production stage after adaptation to production media during two passages. The batch production supernatant was harvested after cultivation for 4 days without any additional feeds.

2.6 Antibody analytics in supernatant

Supernatants were clarified (1000 g, 30 min, 4°C centrifugation and 1.2-μm filtration, AcroPrep 96 Filter Plates, Pall Cooperation). Analytical protein A chromatography was performed by ultra high performance liquid chromatography with ultraviolet (UV) light detection (Bionex Ultimate 3000 UHPLC fitted with POROS™ A 20 μm Column, Thermo Fisher Scientific Inc.). Antibody integrity was analyzed after protein A affinity chromatography (PreDictor RoboColumn MabSelect SuRe, Cytiva) and normalization with protein quantitation using UV measurement (NanoQuant Infinite M200, Tecan). Percentage of correctly assembled antibodies (Main-Peak) was assessed by capillary electrophoresis sodium dodecyl sulfate (CE-SDS; HT Antibody Analysis 200 assay on the LabChip GXII system, PerkinElmer) under non-reducing conditions by relative quantification of the expected protein size to total protein content.

Antibody quality was analyzed by liquid chromatography–mass spectrometry (LC-MS) as described by Haberer et al. (27). Poros A purified antibody material was denatured in 0.3 M Tris, 2 mM ethylenediaminetetraacetic acid and 6 M Gua, pH 7. For reduction, 4 μl of 1 M dithiothreitol (DTT, Roche) was added, followed by incubation at 37°C for 1 h. After alkylation of free cysteines by adding 10.4 μl of 1 M iodoacetic acid and incubation at RT in the dark for 15 min, the reaction was stopped by adding 2 μl of 1 M DTT. The buffer was exchanged to digestion buffer (50 mM Tris/HCl and 2 mM CaCl2, pH 7.5) by application onto a NAP-5 gel filtration column (GE Healthcare). Subsequently, the NAP-5 eluate (500 μl) was mixed with 10 μl of a 0.25 mg/ml trypsin solution (Trypsin Proteomics grade, Roche) in 10 mM HCl and incubated at 37°C for 1 h. The digest was stopped by adding 10 μl of 54 mM Met in 80% formic acid in water. Ten microliters of the tryptic peptide mixture were injected for analysis.

Samples were separated on a RP C18 column (BEH C18 1.7 μm, 2.1 × 150 mm; Waters) using an UltiMate 3000 Rapid Separation LC (Thermo Fisher Scientific Inc.) and analyzed online with an Orbitrap Fusion Trivibrid electrospray mass spectrometer (Thermo Fisher Scientific Inc.). The mobile phases consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The chromatography was carried out using a gradient from 1 to 35% Solvent B in 45 min and finally from 35 to 80% Solvent B in 3 min using a flow rate of 300 μl/min. UV absorption was measured at a wavelength of 220 nm. The Rapid Separation Liquid Chromatography system and mass spectrometer were connected by PEEK capillary tubing. Data acquisition was controlled by the Orbitrap Trivid MS Series Instrument Control Software Version 3.4 (Thermo Fisher Scientific Inc.). Parameters for MS detection were adjusted according to existing knowledge gained from experience with peptide analysis of recombinant antibodies.

2.7 RNA sample preparation and analysis

Control and KO pool cells (10 × 10^6) sampled from the ambr15® bioreactor on Day 10 were washed twice in PBS and snap-frozen in liquid nitrogen. RNA extraction, Illumina stranded TruSeq RNA library preparation, poly(A) enrichment and sequencing (NextSeq, v2.5, high output 1 × 75 bp) was performed by Microsynth AG.
Figure 1. Arrayed CRISPR-screening-workflow identifies Myc KO as beneficial KO for productivity. (a) RNP-based pooled single-gene KO protocol, using three sgRNAs binding in close genomic proximity and targeting the landing pad-encoded GFP in the TI-CHO host cell line. After GFP KO, cells were grown for 6 days and analyzed using flow cytometry. (b) Experimental outline of the pooled single-gene KO strategy, exemplary shown for Myc, Stk11, Dot1l, Smad4, Sqstm1 and Ppp2cb gene KO. (c) Experimental high-level outline of the arrayed CRISPR/Cas9 screening process. Key target key players of different cellular pathways including cell growth, protein secretion, stress responses and others were knocked-out using CRISPR/Cas9, and CHO cells were tested in a production-mimicking batch process. (d) Normalized bsAb titers after protein A purification from the arrayed CRISPR/Cas9 KO screening using a production-mimicking 4-day batch process.

(Belgach, Switzerland). Sequences for the transgene and the reference genome were analyzed separately. Reads were aligned using the HISAT2 package (version 2.2.1), and transcript abundance was calculated with featureCounts (version 2.0.1). The percentage of reads that mapped to either the transgene or the reference sequence ranged from 80.7 to 90.2%. Differential expression was calculated using edgeR by pooling untreated and Myc KO production clones as biological replicates. Significant changes in expression level were defined based on P-value (P < 0.05) and fold change (|FC|> 1.25) cut-offs. Over-representation analysis (ORA) was performed on all significantly changed genes by g:profiler (https://biit.cs.ut.ee/gprofiler/).

3. Results

3.1 An arrayed CRISPR/Cas9 KO screen reveals Myc KO to increase complex bsAB productivity

CRISPR/Cas9-based gene KOs are the preferred choice for simple, fast and efficient perturbation of target gene functions. To be able to evaluate the effect of any given KO in an efficient and unbiased manner, we established a highly-efficient RNP-based multi-guide pooled single-gene KO strategy for a TI-CHO cell line (10). Our optimized RNP-based KO protocol was established by targeting the landing pad encoding green fluorescent protein (GFP) in the TI-CHO host cell line using three sgRNAs binding in close genomic proximity and resulted in a complete loss of GFP fluorescence 6 days after RNP delivery (Figure 1a, Supplementary Figure S1a). Utilizing this pooled single-gene KO strategy (selected examples are shown in Figure 1b), we rationally chose 187 key players of different cellular pathways including cell growth, protein secretion, stress responses and others as target genes for KO (Figure 1c and Supplementary Table S1). To test the effect of each KO on productivity, we subjected all single-gene KO pools to a 4-day batch production process, which mimics the conditions used in state-of-the-art bioprocesses. We verified that variation of product titer within our screening system was limited to <3% of standard deviation (Supplementary Figure S1b).
KO of these target genes in two CHO clones revealed that pooled KO of Dot1l, Stk11, Cdk12, Sgstm1, Smad4 and Ppp2r2b improved productivity of both clones by >10% in a batch process (Figure 1d). KO of conditionally essential genes Rbx1, Vhl, Wee1 and Chk1 and KO of the essential gene Map3k7 resulted in loss of cell population after KO in one or both tested CHO clones, confirming the highly-efficient KO protocol (28) (Figure 1d). PCR amplification of our multi-guide target sites within the genes revealed substantial locus fragmentation compared to the unmodified locus, exemplarily showed for Myc, Stk11, Dot1l, Smad4, Sgstm1 and Ppp2r2b (Supplementary Figure S1d). We found no significant differences in main product quantities as measured by CE-SDS of protein-A-purified supernatants, indicating that the correct assembly of the antibody chains was not perturbed by the introduced KOs (Supplementary Figure S1c). Intrigued by the strong impact of Myc KO on productivity, we decided to focus on its validation across different cell lines and molecule formats.

3.2 Myc KO reduces cell proliferation and consistently improves SPRs in PSB clones

Next we wanted to exclude the option that the observed Myc KO phenotype is an artifact of the two tested CHO clones. We, therefore, tested Myc KO and respective control cells performance side-by-side on seven different CHO clones from a primary seed bank (PSB) of M1 for production performance under fed-batch conditions in ambr15 bioreactors, an important step for the selection of lead clones during cell line development (Figure 2a). All Myc KO pools showed a volumetric productivity increase between 7 and 29% compared to control cells (Figure 2b) and no loss in product quality, as assessed by CE-SDS analysis (Figure 2c). Simultaneously, we observed a substantial decrease in viable cell density (VCD) during the bioprocess, displayed by a 28% reduced peak VCD at Day 7 (Figure 2d). This is in line with previous findings where depletion of Myc resulted in the inhibited proliferation of tumor cells (29). Cell division ceased around Day 7 during the fed batch with peak VCD in general reached on Day 7. However, cells continued to grow in volume and increased their average diameter by 3μm, which amounts to 2-fold between the start and end of the fed batch (Figure 2e). Myc KO pools showed an additional 15% increase in cell volume on average compared to control cells, indicating a partial shift from cell division toward cell growth by Myc KO (Figure 2e).

To rule out that the phenotype observed after Myc KO was the result of undesired locus fragmentation, we generated Myc KO with individual sgRNAs instead of the previous multiplexing approach. Using multiple sgRNAs simultaneously can lead to unintended off-target effects as for example rearrangements in the genome that affect protein production. We tested Myc ablation with three different sgRNA sequences independently using two different producer clones during fed-batch production (Figure 1b). Notably, all three sgRNAs target the coding sequence of Myc, but share no sequence similarity and have different predicted off-target sequences (Supplementary Table S4). All three used sgRNAs improved volumetric productivity in both CHO clones as well as decreased peak VCD during fed-batch production (Supplementary Figure S2c and d).

We conclude that Myc KO improved volumetric productivity consistently in CHO clones, potentially through a general switch from growth toward production phenotype.

3.3 Myc KO improves specific productivity regardless of the molecule format

Next, we evaluated whether Myc KO improves productivity of other CHO clones expressing various complex molecule formats in an MBR set-up. We selected eight antibody-producing PSB candidates each producing a distinct complex format (Figure 3a). M1: Cytokine Fusion, M3/M9: 1+1 CrossMab, mAB, M4: mAB, M5: DutaFab, M6: Brain Shuttle Fusion, M7: 2+1 T-Cell Bispecific, M8: Fc-Fragment). To enable KO quantification by Sanger trace decomposition, we reduced the amount of sgRNA to one and observed the predicted Myc KO efficiencies between 13 and 80% in the production clones (Supplementary Table S2). We, therefore, expected an intermediate impact on productivity phenotype; however, by conducting experiments in a pool format we could simultaneously exclude any potential impacts that would otherwise be introduced via sub-cloning process. We analyzed all Myc KO and respective control cells side-by-side for production performance using an industrial-relevant fed-batch process in ambr15 bioreactors. Of note, production clones display substantial clonal heterogeneity and therefore show increased variation in general cell characteristics (14).

Remarkably, all Myc KO pools showed improved volumetric productivity in most cases immediately after the beginning of the production process (Figure 3b). On average, the Myc KO pools showed a 17% reduction in peak VCD, indicating that Myc supports cell division during fed-batch production (Figure 3c). In general, cell division ceased quickly during the fed batch with peak VCD reached on Day 3. However, cells continued to grow in size and increased their average diameter by 3–5μm between the start and end of the fed batch in Myc KO conditions (Figure 3d). Myc KO pools showed an additional 8% increase in cell size compared to control cells throughout the fed batch, indicating a partial shift from cell division toward cell volume growth upon Myc KO. The increased overall product is based on higher SPRs of Myc KO pools during the whole process: on average SPR increased between 14 and 99% (Figure 3e).

To verify that Myc KO led to the accumulation of high-quality product we tested the overall protein composition as well as post-translational modifications (PTMs) of each molecule. Increased protein production puts additional load on the secretory pathway that, in turn, might affect folding and PTMs. CE-SDS analysis of Myc KO pools and controls demonstrate highly comparable overall molecule composition (Supplementary Figure S2a). Additionally, we analyzed Fc glycosylation, Asn deamidation, Met oxidation, Lys-loss, and N/C-terminal modifications at the peptide level by LC-MS. We did not find any significant alterations on the PTM pattern on molecules produced in Myc KO cells, as compared to the control (Supplementary Table S5).

To evaluate the molecular basis for increased SPR of Myc KO cells, we analyzed the CHO cell transcriptome by RNA-Seq at Day 10 of fed-batch production. Depending on the production clone, between 8 and 29% of the transcriptome was derived from the antibody transgenes (Figure 3f). This percentage increased on average by 3%, indicating that a generally higher portion of Myc KO cells’ transcriptome contained the antibody transgenes. Upon transcript normalization, Myc KO cells...
Figure 2. The beneficial effects of Myc KO are consistent among all tested clones of a bsAb PSB. (a) Schematic overview of an industrial cell line development process. Stable expression pools are tested for pool performance, and high-productivity pools undergo single cell cloning. Clones are expanded, banked (PSB) and tested for clone performance in ambr15 bioreactors. After upscale and validation using ambr250 bioreactors, the best-performing PSB clone will be expanded as the master cell bank. Illustration created with BioRender.com. (b) Day-14 titers of ambr15 fed-batch process after protein A purification. Myc KO pools and mock-treated control cells of seven high-productivity PSB clones expressing a cytokine-fusion bsAb (M1) are shown. (c) Day-14 product quality (main peak, CE-SDS) of ambr15 fed-batch process of Myc KO pools and mock-treated control cells of seven high-productivity PSB clones expressing a cytokine-fusion bsAb (M1) are shown. (d) VCD and (e) average diameter of individual cells during the 14-day ambr15 fed-batch process. All data are mean ± SEM. P = adjusted P-values. Statistical significance was determined using the Holm–Sidak method. Each row was analyzed individually, without assuming a consistent SD.

showed 22–32% increase in relative antibody transcript abundance (Figure 3g). This indicates that the enhanced productivity phenotype of Myc KO cells may result from increased expression levels of antibody chain-encoding transcripts. ORA further showed that Myc depletion resulted in the enrichment of kyoto encyclopedia of genes and genomes pathways’ protein processing in the
Figure 3. Myc depletion improves specific productivity of various CHO clones regardless of the expressed complex molecule format. (a) Schematic drawing of the individual molecule formats. All CHO clones resulted from individual industrial-relevant cell line developments. (b) Product titer of ambr15 fed-batch process after protein A purification. Simultaneous assessment of (c) VCD and (d) average cell diameter. Production clone performance of mock-treated control cells compared to Myc KO cells. (e) Specific production rates over the course of the ambr15 fed-batch process comparing mock-treated control cells with Myc KO cells. Specific production rates were calculated based on the product titers after protein A purification and the integrated cell volume over time. (f) Fraction of reads mapped to bsAb transgene sequences out of all reads that were successfully mapped to the Chinese Hamster RefSeq genes. All transgene chains were included in the analysis. Data from Day 10 of the ambr15 fed-batch process. (g) Normalized transcript counts from Myc KO cells mapping to one of the bsAB transgene chains relative to mock-treated control cells. Data from Day 10 of the ambr15 fed-batch process.
endoplasmic reticulum (ER) and protein export (Supplementary Table S3). Simultaneously, transcripts associated with the ER in general are significantly upregulated in Myc-depleted cells. This suggests that Myc KO further improved productivity of CHO clones by the increased expression of ER-related pathways.

These data indicate that Myc KO improved production performance of CHO cells in industrially-relevant production processes, regardless of the type of therapeutic protein and without impacting product quality.

### 3.4 Myc KO cells reliably improve production performance in scale-up ambr250 bioprocesses

The successful application of Myc KO cells in commercial scale processes requires the use of a reliable and qualified scale-down model (SDM). The ambr15 system is only suited as a SDM within bench scale (up to 200-1 stirred bioreactors) (19, 30). To replicate the fermentation results within commercial scales, we next evaluated the performance of Myc KO in a scale-up fed-batch bioprocess using the ambr250 system (31). The higher cultivation volume (200–250 ml) compared to 10–15 ml in the ambr15 allowed for increased sampling and product profiling, which otherwise would result in the substantial loss of production biomass in the ambr15 system. To verify the Myc KO in the ambr250 system, we generated Myc KOs in two lead industrial-relevant production cell lines (M1 and M2, Figure 4a and b).

Immediately after starting fed-batch production, Myc KO cells displayed increased volumetric productivity resulting in 38–41% increased titer on Day 14 of the fed-batch process. bsAb titers increased by 2067 and 2128 mg/l, respectively, with SPR of the Myc KO cells constantly higher throughout the process and in both cell lines by 8–23 pg/(cell × d). Peak VCD was reached on Day 3 and decreased steadily afterward, with Myc KO cells reaching lower VCD throughout the production process. After cells ceased to divide, cell growth continued in size with all conditions increasing their average diameter by 4–6 μm until the end of the fed-batch process. Myc KO cells showed an additional 14–16% increased size compared to control cells. The product profile from cell line molecule M1 (Cytokine Fusion) was assessed by CE-SDS and size-exclusion chromatography and showed comparable main product and aggregation rates in the supernatant after protein A purification (Supplementary Figure S2b, left panel). The product profile from cell line molecule M2 (1 + 1 ligand binder) displayed slightly reduced main product levels (Supplementary Figure S2b, right panel).

Together, our findings indicate that Myc KO cells displayed a superior phenotype during ambr250 fed-batch production by further boosting specific and volumetric productivity of clones in an industrially-relevant production process.

### 4. Discussion

Despite decades of improvements in CHO bioprocesses, high-value DTE bsABs still challenge current production capacities. To generate a high-yield expression system, genetic modifications of the expression system are increasingly used to improve their sub-optimal secretory phenotype. Previous studies have shown that the productivity of DTE antibodies can be enhanced by decoupling growth and production phases (32–34). This results in the downregulation of cell proliferation and a redistribution of cellular resources toward increased recombinant protein production (35). Common strategies to halt proliferation and increase productivity included mild hypothermic cultivation (36), osmotic stress (37), addition of chemical agents (38) or genetic engineering (34).

However, not all cell lines with high productivity show an increase in productivity by lowering temperature or increasing osmolarity during production, suggesting that these strategies are product, cell line or even clone specific (39, 40). In this study, we therefore decided to apply an unbiased screening approach by introducing CRISPR-induced KOs into CHO clones expressing DTE complex antibody formats and measured productivity increase side-by-side to the non-engineered parent clone. We identified Myc KO to consistently increase productivity of multiple CHO clones expressing different DTE formats in industrially-relevant bioprocess applications. In addition to the superior production phenotype, Myc KO cells exhibit reduced cell growth, increased cell volume and substantially improved SPRs.
The transcriptomic analysis of Myc KO cells demonstrates a substantial increase of antibody-encoding transcripts in fed-batch production processes. Thus, the improved specific productivity upon Myc depletion appears to be, at least partly, related to the observed higher mRNA levels. Studies using a range of cell lines suggest that higher transgene mRNA levels contribute to improved specific production rates until a certain saturation level of mRNA, after which the control may shift to post-translational events (41). Here, we found that CHO clones devote up to 29% of their total mRNA to the transgene, which is increased by ~4% on average by Myc depletion. Interestingly, the relative transcript count shifted to a much higher proportion toward the transgene, resulting in a relative increase in transgene transcript levels by ~25%. Thus, Myc depletion redistributed transcriptional resources toward the transgene transcription. It is conceivable that the strong gene expression elements used in the transgene attract the freed transcriptional resources previously spent on cell division and thus increase the relative abundance of transgene mRNA. These transcriptional changes were associated with higher expression of ER-related proteins. Myc depletion seems to impact both transgene transcription and translational machinery of CHO clones and extends their overall production capacities. This observation was supported by the findings that while all the Myc KO clones expressing different molecules showed increased specific productivity, two did not increase their transgene mRNA levels.

A similar phenotypic change, i.e. the shift from reduced cell proliferation toward increased cell volume and higher specific production rates, was observed in a recent study in CHO production cells when overexpressing the BLIMP1 gene (42). The authors associate the effects of BLIMP1 overexpression with the repression of Myc levels, indicating a similar biological mechanism to direct Myc ablation. However, in contrast to our study, no increase in transgene mRNA levels was observed after BLIMP1 overexpression (42). Myc as a direct engineering target has been addressed in different contexts before, due to its general role in cell proliferation and apoptosis. For example, Myc overexpression in CHO cells was previously shown to result in increased cell densities and growth rates (43). In contrast, the suppression of Myc results in inhibited cell proliferation, together with G1/G0 cell cycle arrest and decreased apoptosis (44). Due to the strong effect of Myc KO on cell proliferation, cells with intact endogenous Myc will proliferate faster compared to their KO counterpart. This may explain the observed varying levels of Myc KO levels in the different CHO clones.

Utilizing multiple KOs could further amplify the beneficial effect observed with Myc KO. This could especially be the case if the productivity is increased by different mechanisms, e.g. increasing transgene transcript abundance while simultaneously alleviating secretory bottlenecks during folding or secretion. Kol et al. have shown that this multiplexed engineering approach can modify multiple cellular characteristics simultaneously, i.e. reducing unnecessary host cell proteins while simultaneously increasing productivity and cell growth (20). Future studies could combine the partially beneficial effects we observed after Dot1L, Szk11, Cdk12, Sostm1, Smad4 and PPP2CB KO in combination with Myc KO.

Finally, it is worth noting that the effects of Myc ablation on productivity might synergize well with high-cell density production processes, where the higher SPRs together with increased cell densities lead to substantially improved volumetric titer. In these production conditions, the inhibition of cell proliferation has lesser impact on volumetric titer, as these processes start with cell densities of 10–20 × 10⁶ cells/ml or more. In summary, the data provided in this manuscript illustrate the potential of CRISPR/Cas9-based CHO host cell engineering and provide an avenue to increase manufacturing yields of high-value complex biotherapeutics.

**Supplementary data**

Supplementary data are available at SYNBIO online.

**Data availability**

The data supporting the findings of this study are available within the article and its supplementary materials. Nucleic acid sequences encoding for antibody sequences are proprietary to Roche.

**Material availability**

CRISPR/Cas9 gRNAs are available upon request. Nucleic acids and cell lines encoding for antibody sequences are proprietary to Roche.

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**Author contributions**

N.B., B.O., M.E., L.S., E.L. and A.O. designed, performed and analyzed the experiments. S.A. conceived the project and obtained funding. S.A., C.S., A.S., S.M. and J.S. interpreted the results and helped in improving the manuscript. N.B. wrote the manuscript with input from all authors.

Conflict of interest statement. A patent based on this work has been filed with authors NB, B.O. and S.A. as inventors. NB., B.O., M.E., L.S., E.L., C.S., A.O., A.S., S.M. and S.A. were employees of Roche Diagnostics GmbH or Genentech, Inc., which developed and sold pharmaceuticals during the time when this research was carried out. All other authors declare no competing interests.

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