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Towards a Whole Genome KO Screen in CHO DG44 to Unravel the Link Between Genomics and Fast Growth Phenotype

Jannis Marzluf[1,2], Ann-Cathrin Leroux[1], Christoph Zehe[1]
[1]Sartorius Stedim Cellca GmbH, Ulm, Germany // [2]Department of Gene Therapy, University Hospital Ulm, Germany

jannis.marzluf@sartorius.com

Background

Chinese Hamster Ovary (CHO) cell lines are extensively utilized in biopharmaceutical production. Improvements in cell line generation have accelerated the speed to final production clones, yet challenges in developing novel biomolecules, productivity limitations, and market demands necessitate continuous improvement in cell line development (CLD). While cell growth displays a significant bottleneck during CLD, limited research has focused on the growth phenotype of CHO cell lines. A recent study successfully isolated and immortalized a novel Chinese hamster cell line derived from primary lung cells, exhibiting faster growth rates, stable productivity, and high levels of biopharmaceutical protein production[1]. Notably, the CHL-YN cell line demonstrated an improved doubling time of 10.7 h, compared to the typical 18.0 to 22.0 hours observed in CHO cell lines. Here, we aim to conduct a comprehensive whole genome knockout (KO) screen to identify genetic targets and pathways that accelerate CHO cell growth uncovering the fundamental genetic mechanisms associated with CHO cell growth. We have established a robust CRISPR competent CHO DG44 cell line, capable of producing Insertion-/Deletion-(InDel) events in a predictable manner in the presence of single guide RNAs (sgRNAs). Furthermore, we tested our methodology for the generation and cultivation of a transduced library with a small 140 sgRNA minilibrary. Our optimized setup was able to achieve around 80% single copy integrations, an improvement over past works from recent literature[2]. Additionally, we provide evidence for CRISPR-nuclease expression dependent efficiency of enrichment and depletion for gene targets that affect growth.

1 A Robust CRIPSR Competent Cell Line

Outlined Requirements for stable CRISPR nuclease expressing CHO cell lines:

- Capable of producing CRISPR editing events with **high efficiency** in stable generated CHO DG44 pools after transient or stable delivery of sgRNAs targeting putative genes
- Demonstrate **temporal stability** of genetic editing outcomes in stable CHO DG44 pools for the duration of the screen (>six weeks)

» Tasks: Generation of stable CHO^{Cas+} cell lines with multiple bicistronic Cas expression vectors for coupled expression by transfection and G418 selection with subsequent evaluation by stable and transient sgRNA delivery at different time points

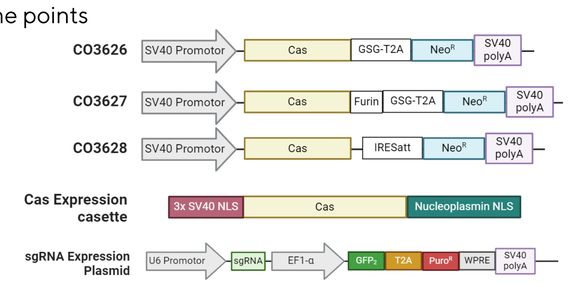


Figure 1: **Schematic representation of vectors used for expression of Cas in CHO DG44 cells.** CO3626: Bicistronic expression with GSG-T2A linker coupled to neomycin resistance gene. CO3627: Similar setup to CO3626 but with added Furin recognition site (RRKRR) site upstream of GSG-T2A linker. CO3628: bicistronic vector with 5'cap independent expression of neomycin resistance gene. Mutated internal ribosomal entry site (IRES) of encephalomyocarditis virus (EMCV) mediates low-efficiency ribosomal binding. Expressed Cas has three N-terminal SV40 Nuclear localization signal (NLS) and one C-terminal Nucleoplasmin NLS. The sgRNA expression cassette used for testing of stable Cas expressing cell lines and lentiviral transduction is also shown.

Key takeaways from stable expression of an sgRNA in stable Cas expressing Pools:

- **High KO-Scores** achieved
  - Lead construct 3627 showed KO-scores of 98.0 and 96.5 in both replicates
- Selection worked as intended with >99.0% of cells expressing the GFP-Marker 15 days after Transfection

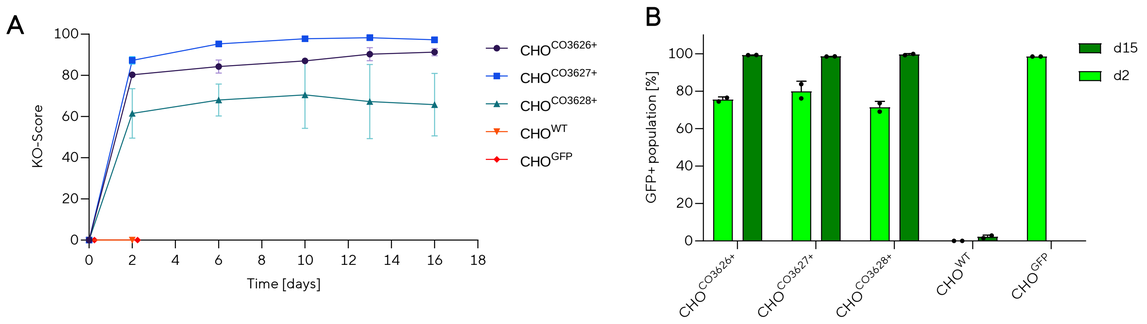


Figure 2: **KO-scores after transfection with psgRNA6 encoding an sgRNA targeting FUT8 into stable Cas-pools generated with CO3626/-27/-28.** A: Cas pools were generated in 2 weeks of selection with G418 in SMD media. After selection, the large pools were transfected with sgRNA6 encoded in a plasmid and selected with Puromycin. [n=2 ; Mean ± SD] B: GFP positive ratio after TR and post selection. [n=2; Mean ± SD]

Cas expression may be controlled by antibiotic Gene Amplification and CRISPR competence is retained over at least nine weeks:

- Expression level of Cas **responds to increases in selection pressure** in stable pools in a dose-dependent manner (mean **1.5-fold** for mid cas-level expression and mean **3-fold** increase for high cas level)
- Upon receiving an sgRNA (GFP-Reporter), Cas expressing Pools exhibit KO-score ranging from 80.0 to 99.0, contingent on the expression vector and amplification level
- Generated Cas expressing pools demonstrate **temporal stability** of editing capabilities with high KO-scores achieved across nine weeks of cultivation with and without selective pressure

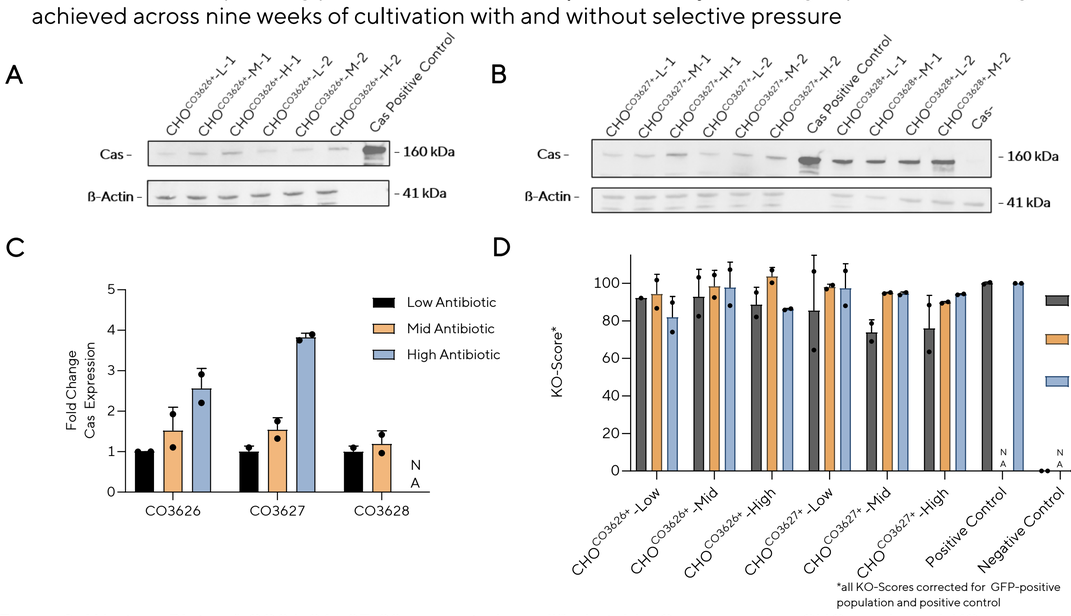


Figure 3: **Western-Blots of CO36-26/-27-28 generated amplified stable Cas expressing CHO pools and KO-scores post selection generated with CO36-26/-27.** A & B: Blots were performed with 100.000 cells each. Annotations describe the construct used for cell pool generation and the amount of antibiotics used for selection. (L= 250µg/mL; M= 750 µg/mL; H= 2000 µg/mL). Two replicates were loaded next to each subsequent concentration ladder. Recombinantly purified Cas is used as positive and untransfected wildtype cells as negative control. C: Fold change in Cas protein amount based on different levels of amplification by G418. Expression normalized against ß-actin. [n=2 ; Mean ± SD] D: Cas pools were generated in 2 weeks of selection with G418 in the amounts described above. After selection the large pools were transfected transiently with sgRNA6 encoded in psgRNA6 and analyzed by PCR amplification of cut sites and flow cytometry, 3 weeks after selection and 9 weeks. [n=2 ; Mean ± SD]

2 Setup of an Optimized Pooled CRISPR Screen in CHO DG44

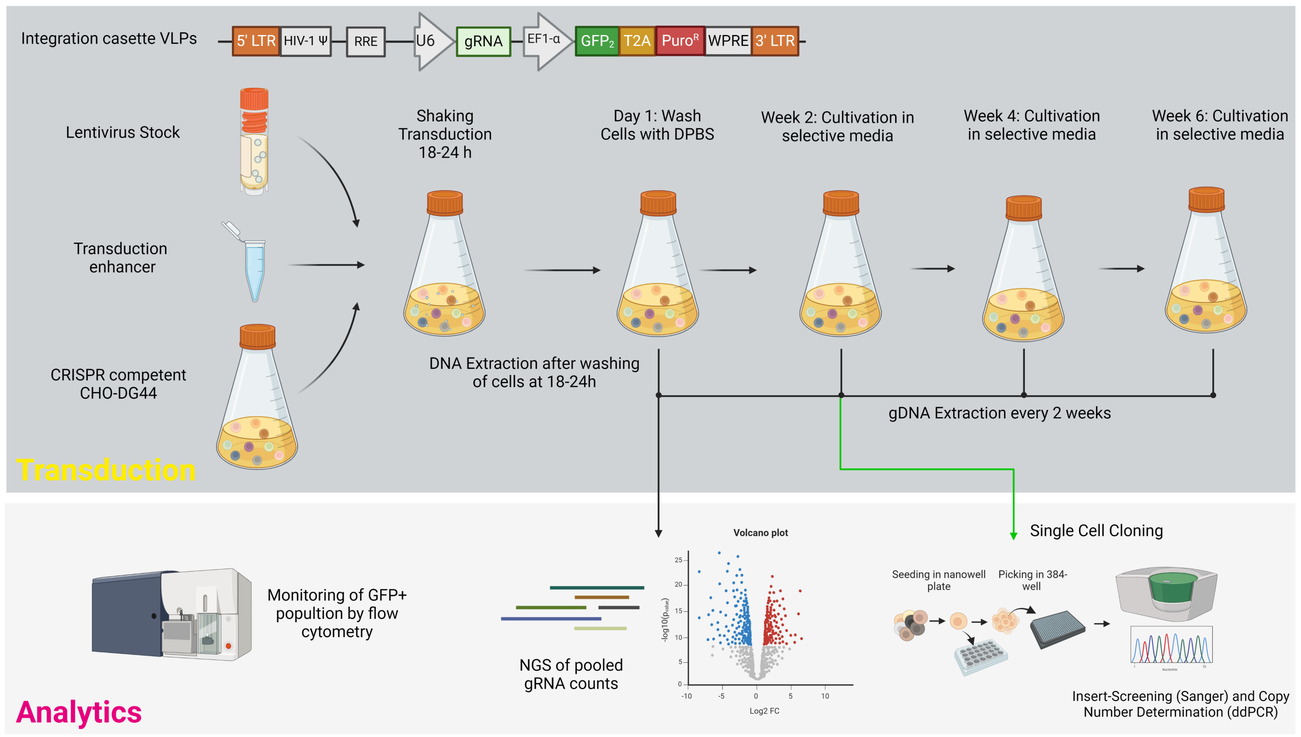
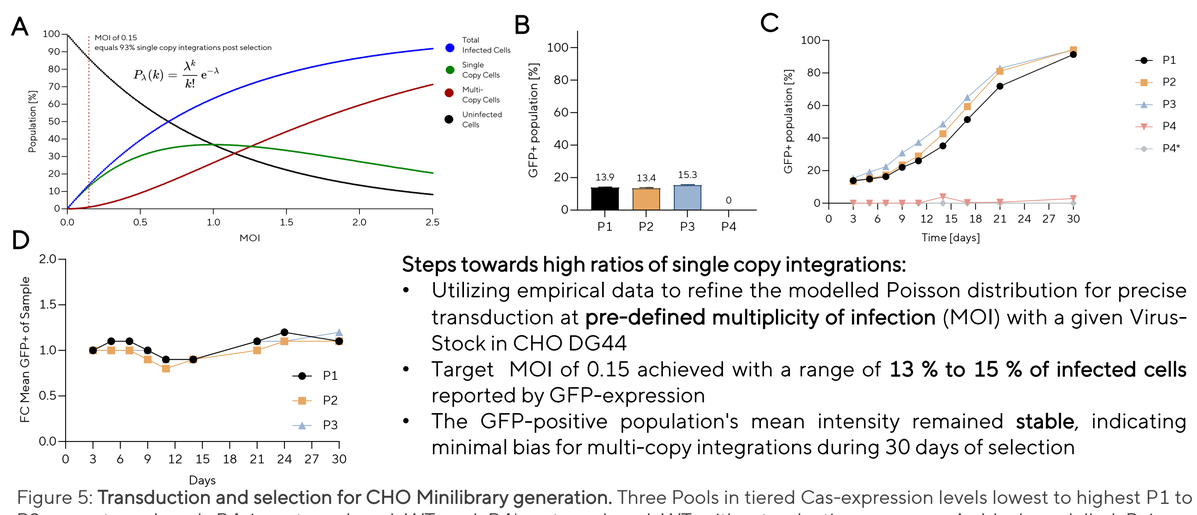


Figure 4: **Workflow for the generation of pooled CHO KO Minilibrary.** Three distinct amplification tiers (Low, Mid & High) of CO3627 generated stable Cas-expressing cell pools were selected as replicates for a preliminary Pilot-Experiment at 5.000x library complexity. Shaking-based transduction with reduced volume at a target MOI of 0.15 was performed. Genomic DNA (gDNA) was extracted one day post-transduction to establish time point zero quantification of sgRNA read counts. Throughout the period of the screen gDNA was extracted bi-weekly to serve as point of comparison. Additionally, Single cell clones were isolated three weeks post-transduction for the analysis of transduced copy-numbers. The gDNA samples were used to prepare a Next Generation Sequencing (NGS) library, followed by Illumina HiSeq sequencing to assemble read counts of all sgRNAs. The transduced population was tracked using flow cytometry and the expressed GFP reporter.

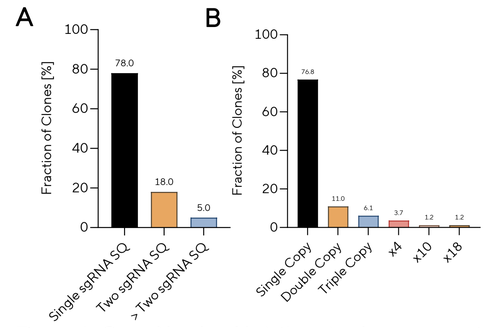
3 Optimized Transduction for Single-Copy Integrations



Steps towards high ratios of single copy integrations:

- Utilizing empirical data to refine the modelled Poisson distribution for precise transduction at **pre-defined multiplicity of infection** (MOI) with a given Virus-Stock in CHO DG44
- Target MOI of 0.15 achieved with a range of **13 % to 15 % of infected cells** reported by GFP-expression
- The GFP-positive population's mean intensity remained **stable**, indicating minimal bias for multi-copy integrations during 30 days of selection

Figure 5: **Transduction and selection for CHO Minilibrary generation.** Three Pools in tiered Cas-expression levels lowest to highest P1 to P3 were transduced. P4 is untransduced WT and P4* untransduced WT without selective pressure. A: Ideal modelled Poisson distribution with annotated actual MOI for Transduction. B: GFP positive cells after Transduction. [n=1; three technical replicates; Mean ± SD] C: GFP positive cells were selected with 5 µg/mL puromycin over a period of 30 days. [n=1] D: Mean fold change in intensity of GFP positive population. [n=1]

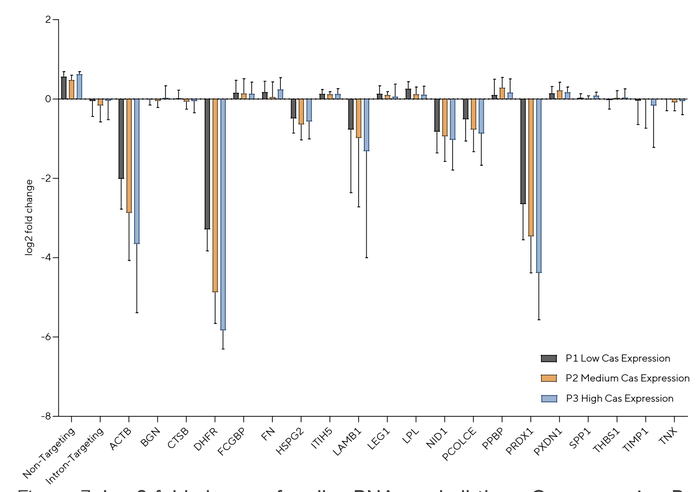


Achieving High Ratios of Single copy integrations:

- Post-selection **single sgRNA integrations** are 78.0 % and 76.8 % based on Sanger-insert-sequencing and ddPCR respectively
- Large improvement to current literature in CHO that showed ~40% single copies integrated[2]

Figure 6: **Copy-Number Variations in clones generated from transduced pools.** A: Integrated sgRNAs analyzed by PCR and Sanger-sequencing of sgRNA expression cassettes. [n=86] B: Copy Number Variations analyzed by ddPCR and normalized against endogenous housekeeping gene. [n=92]

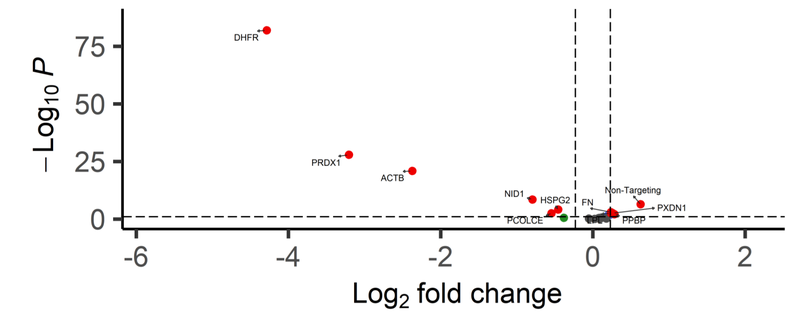
4 Insights from Pooled CRISPR Screen Data



Better Data and increased Temporal efficiency for Target Discovery:

- Cas expression-level impacts CRISPR screen data quality and time needed to accrue hits
- Elevated Cas expression levels coincide with **augmented log2 fold changes** in sgRNA read counts for genes that affect phenotypic selection
- Large benefit of temporal efficiency, hits are identified earlier and with **improved signal to noise ratio**

Figure 7: **Log2 fold changes for all sgRNAs and all three Cas-expressing Pools.** Data derived from DeSeq2 normalized read counts, means calculated for all sgRNAs per target gene/control. [n=10 (Non- and Intron-Targeting); n=6 (all Genes); Mean ± SD]



Higher Performance pooled CRISPR screen :

- High log2 fold reduction in essential genes (DHFR, ACTB, PRDX-1)[3,4,5]
- Signal to noise ratio allows for identification of small effects on growth
- We may **resolve 0.25 log2** fold changes with high confidence

Figure 8: **Enrichment/Depletion analysis of all included genes and controls comparing week six to week two.** Read counts derived from MAGeCK and statistical analysis performed with DeSeq2 normalized read counts. [n=10 (Non- and Intron-Targeting); n=6 (all Genes)]

5 Conclusion & Outlook

In summary, the established pooled CRISPR screening platform enables a comprehensive evaluation of the CHO genome's impact on cell growth. With an unprecedented library coverage of >5.000x, we aim to generate the highest quality data to date, potentially offering opportunities for novel methodologies in the broader field of pooled CRISPR screening in addition to facilitating the engineering of superior CHO DG44 cell lines.

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