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REVIEW ARTICLE

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Systematic review and data-driven insights into CHO cell engineering for next-generation antibody production

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ABSTRACT

Chinese hamster ovary (CHO) cells remain the dominant platform for therapeutic antibody and biopharmaceutical production, yet productivity bottlenecks persist, particularly for complex molecules. To identify overarching trends in host cell optimization, a systematic review and quantitative cross-study analysis of 164 publications (2011–2024) reporting CHO cell engineering strategies with effects on titer or specific productivity was conducted. Data from 466 engineered targets were extracted and analyzed by strategy, pathway, and production context. The field – driven largely by antibody production – has evolved from simple overexpression toward CRISPR-mediated knockouts, while combinatorial approaches, and engineering of nuclear, epigenetic, and apoptotic/proliferative targets achieved the greatest gains. Despite technological advances, reported improvement folds remained stable, highlighting the need for pathway-informed, multi-target engineering. Future progress in predictive modeling of engineering strategies will depend on standardized models and structured datasets. This review provides a data-driven framework for rational CHO design to support next-generation biotherapeutic production.

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Introduction

Since the approval of the first therapeutic recombinant protein, tissue plasminogen activator (tPA) in 1986,[1] both the market and the therapeutic protein portfolio have expanded exponentially, with global sales exceeding USD 340 billion by 2021.[2] Monoclonal antibodies (mAbs), offering new and targeted treatment options for complex diseases such as cancer and autoimmune disorders, have emerged as the most successful and dominant class among biotherapeutics. This trend began with the market introduction of the first chimeric mAbs (abciximab (Reopro®) and rituximab (Rituxan®)) in 1994 and 1997, respectively.[3,4] Building upon this success, antibody derivatives including bi- and multi-specific mAbs as well as antibody-drug conjugates (ADCs) have been increasingly developed and approved for clinical use since 2014, further broadening therapeutic possibilities.[5]

Chinese hamster ovary (CHO) cell lines have become the predominant host system for therapeutic recombinant protein production, due to their ability to produce complex proteins with post-translational modifications (PTMs), along with their robustness, adaptability to suspension culture and resistance to viral infection. Consequently, 89% of mammalian-derived products and 60% of all approved recombinant therapeutics were produced in CHO cells as of 2021.[2] Consequently, over the past four decades, optimizing CHO cell production has been a major research focus, leading to a remarkable increase in recombinant protein titers and specific productivity (qp, e.g., pg/cell/day). While tissue-type plasminogen activator (tPA) expression titers in 1986 reached approximately 50 mg/L[1], industrial scale mAb production today can achieve titers exceeding 10 g/L.[6] This 200-fold increase has been accomplished through a combination of strategies, including bioprocess

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optimization (culture technology, media and feed), development of selection and amplification systems, advances in gene, vector and host cell engineering, supplemented more recently by systems biology, high-throughput omics, and computational modeling approaches.[7] Despite these advances, CHO cells, like other mammalian expression systems remain limited in production yield, achievable cell densities, and cultivation times compared to bacterial or yeast cultures. Enhancing recombinant protein production in CHO cells thus remains a key challenge for cost-effective biopharmaceutical manufacturing.

The publication of the CHO genome in 2011[8] marked a turning point, enabling novel genetic engineering and screening strategies. However, it remains unclear whether these newer strategies have substantially improved CHO cell productivity. Furthermore, a comprehensive understanding of which engineering targets, pathways targeted, or engineering strategies are most effective is still lacking. A comprehensive cross-study analysis compiling the outcomes of numerous individual reports and engineering targets could help address this knowledge gap and inform future CHO cell engineering efforts. Such an aggregated dataset enables the exploration of key questions such as:

- Which CHO cell engineering strategy is most effective in improving recombinant production?
- Are specific pathways or subcellular localizations more conducive to improvement?
- Is it easier to enhance productivity in low-producing versus high-producing CHO cells?

To address these and other questions, we conducted a systematic literature search of studies reporting CHO cell engineering with effects on titer or productivity, covering publications from January 2011 to December 2024. A broad initial search retrieved 3373 publications. Many of these focused on cultivation and purification process optimization, technology development (e.g., screening platforms), or transgene and vector optimization, which were beyond the scope of this review. After title/abstract and full text screening according to predefined in- and exclusion criteria, the dataset was narrowed to 164 relevant publications. From these, predefined key parameters, including engineering target(s) and mechanism(s), targeted pathway(s) and subcellular localization(s), maximum improvement(s) achieved as well as reported titer and/or specific productivities, were manually extracted. Analysis of this dataset revealed a data-driven perspective, as discussed below. Our findings can guide more effective CHO cell engineering in the future – an important endeavor with the increasingly pressing need given the emergence and clinical adoption of complex, difficult-to-express (DTE) recombinant proteins, including multi-specific mAbs.

Methods of bibliographic compilation and analysis

Our methodology followed the principles of a systematic review, a well-established approach for the comprehensive identification of relevant literature, commonly used in clinical research.[9] The process comprised three main phases:

(1) Identification of relevant databases, development of a comprehensive search string for each database and execution of the search;
(2) Title and abstract screening based on predefined inclusion and exclusion criteria;
(3) Full text review of the identified publications and selection of the relevant literature.

To ensure broad coverage and minimize the risk of missing relevant studies, the search string was deliberately designed to be inclusive, capturing all potentially relevant fields and topics. The complete search and screening workflow is illustrated in Supplementary Figure S1A and described in detail below.

Identification of publications

The following three databases were searched for this review: Medline via Ovid (PubMed), Embase via Ovid (Elsevier) and "Web of Science™ Advanced."

The comprehensive search string was structured around three core concepts: 1) CHO cells, 2) recombinant biopharmaceutical protein production, and 3) cell engineering. For each concept, a set of keywords was defined, which were combined using "OR" operators, while the three concepts were combined using "AND" operators. Both controlled vocabulary (MeSH® terms of Medline, Emtree® of

Embase) and free-text title and abstract queries were incorporated for the keywords in all three databases. The complete search strings including all keywords words are provided in Supplementary Table S1.

Screening and selection of publications

Inclusion and exclusion criteria were defined prior to conducting the literature search. Included studies reported on gene or RNA overexpression, host gene silencing or knockout, engineering of PTMs, or screening approaches evaluating effects on CHO cell productivity. Publications that did not report on recombinant protein production in CHO cells, or focused on process optimization, purification, technology development, or optimization of vector design and/or recombinant protein coding sequence were excluded. Further details are described in Supplementary Figure S1B.

Literature screening and selection steps were performed using Covidence, a web-based collaboration software platform, designed to streamline systematic and other literature reviews.[10] Titles and abstracts were – in a first screening round – assessed for relevance according to the predefined criteria. Full texts were retrieved for publications that passed this title and abstract screening. These full texts were then evaluated in a second screening round for reporting an effect on CHO production titer or qp.

Key performance data extraction

For all included publications and for each engineered target protein and/or RNA reported key performance data, including information on up to 20 pre-specified parameters, were extracted manually. These included the improvement fold(s) achieved, titer(s) and/or qp(s), engineering mechanism, target gene/RNA origin, targeted pathway/mechanism, subcellular localization, the recombinant protein produced, production and engineering settings (transient, stable), publication year, and author affiliations. The full list of key performance data types extracted is provided in Supplementary Table S2. For publications reporting on multiple engineered targets, data were extracted for each individual and (where applicable) combinatorial engineering approach. In cases where numerical values for productivity improvements were not explicitly stated, titers, specific productivities, and/or improvement folds were manually derived from figures by measuring reference and data points and calculating the respective values. One report with an official publication date in 2025 was identified but, for the purpose of time-based analyses, was classified under the 2024 dataset.

Statistical analyses

All statistical analyses and visualizations (box and violin plots) were performed using *GraphPad Prism* version 10.3.0. Tukey style boxplots were used, where the top and bottom of the box represent the 75th and 25th percentiles, the line inside the box indicating the median and whiskers extending to the smallest and largest data points within ±1.5 times the interquartile range.

For multiple-group comparisons, the Kruskal-Wallis test was used. When statistically significant differences were found, a Dunn's test with Bonferroni correction for multiple comparison was applied for pairwise comparisons. For independent two-group comparisons, the Mann-Whitney test was used, while paired data were analyzed using the Wilcoxon matched-pairs test. Statistical significance was defined as $p \leq 0.05$.

Results

Literature search and selection of publications on CHO cell engineering with an effect on production

The initial list of publications retrieved from the three database searches – Medline, Embase and Web of Science – conducted in December 2024 without temporal restrictions included 9521 entries (Supplementary Figure S1A). After de-duplication 5627 unique records remained. For feasibility reasons, a temporal cutoff was necessary. The publication of the CHO K1 genome sequence in 2011 marked a critical milestone,

enabling the development of novel engineering approaches. As we aimed to focus on CHO cell engineering efforts since that breakthrough, the year 2011 was selected as the cutoff date for inclusion. This refinement reduced the dataset to 3373 records. Title and abstract screening identified 1105 publications, meeting the predefined inclusion criteria (described above) and which were selected for full-text screening because they reported on CHO cell engineering (Figure 1). Publications that reported outcomes unrelated to titer or productivity, such as glycosylation modifications, or were protocol-oriented papers without primary data were excluded. The screening led to the identification of 164 original publications describing CHO host cell engineering approaches with effects on productivity. From these 164 reports key performance data were manually extracted for each engineering target reported ($n = 466$). The full dataset is provided in Supplementary Table S3.

Characteristics of the dataset: recombinant protein produced, engineering mechanism, targeted pathways and improvement Fold reported

Recombinant protein produced

Over the past two decades recombinant mAbs have dominated biopharmaceutical production due to their clinical success in treating human diseases such as cancer and autoimmune disorders. More recently, antibody derivatives including Fc-fusion proteins, bi- and multi-specific antibodies as well as ADCs have gained significance, expanding therapeutic options even further.[2] Other therapeutic proteins produced in CHO cells include blood factors like erythropoietin (EPO) and (tPA). This dataset of 164 publications reflects that trend with approximately two-thirds reporting on mAb production (62%, $n = 102$), while about one fifth report on the production of other therapeutic proteins (18%; $n = 30$). Smaller subsets reported on the production of Ab derivatives and "other reporter proteins" like GFP, SEAP or luciferase (10%, $n = 17$ and 9%, $n = 15$), Figure 2(A).

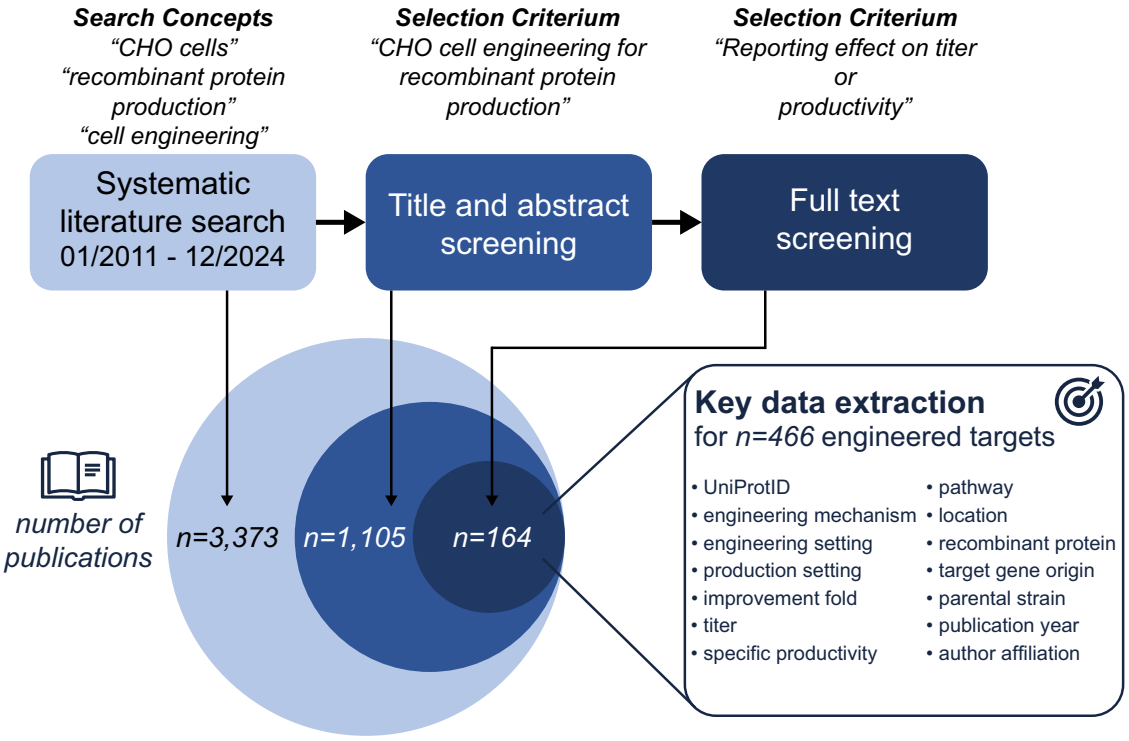

Figure 1. Workflow of the systematic literature search, publication screening, and metadata extraction. The search strategy was structured around three concepts. Search strings were tailored for each of the three databases using a combination of controlled vocabulary and free-text queries in titles and abstracts. Prespecified in- and exclusion criteria were applied during both the title/abstract and full-text screening phases, resulting in the identification of $n = 164$ publications describing CHO cell engineering interventions with measurable effects on titer and/or productivity. From these studies, metadata on production systems, engineering strategies, and publication characteristics were manually extracted for a total of 466 engineering targets.

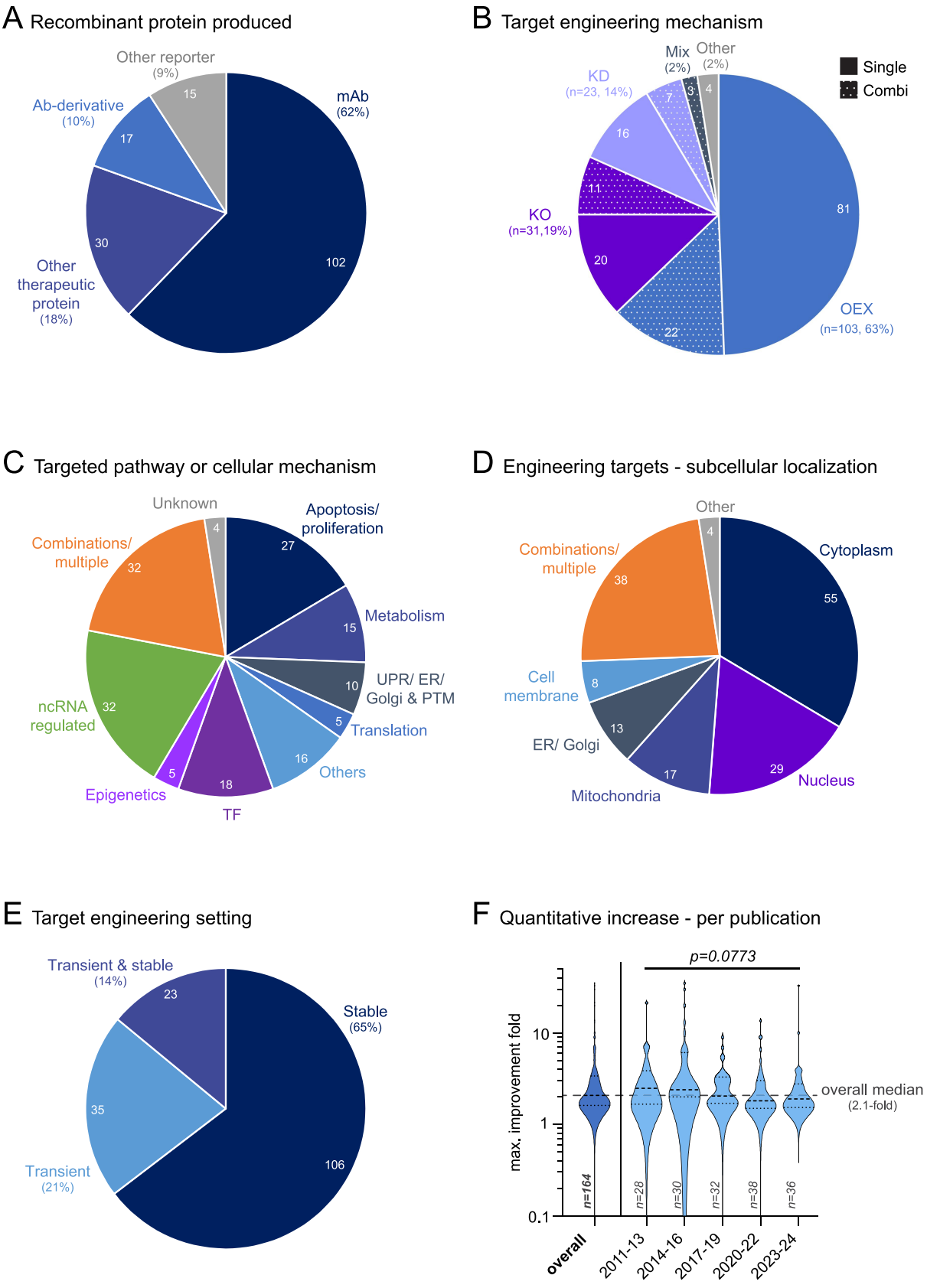

Figure 2. Characteristics of the 164 publications identified in this review on CHO cell engineering for improved production. (A) Distribution of reported recombinant proteins produced. "Ab- derivative" includes Fc-fusion proteins, bispecific antibodies and single chain diabodies. "Other therapeutic protein" refers to products such as blood factors (e.g., EPO), growth factors and enzymes. Reports on the production of classical research reporter proteins including GFP, SEAP or

Target engineering mechanism

CHO cell engineering employs multiple molecular strategies to enhance production, including overexpression (OEX) of beneficial genes, silencing/knockdown (KD) and the complete removal/knockout (KO) of unfavorable targets. In this compiled dataset, the most commonly reported method was OEX in 63% (n = 103) of publications, either by single target OEX (n = 81) or combining OEX of multiple targets (n = 22), Figure 2(B). KO and KD techniques were used less frequently and reported in 19% (n = 31) and 14% (n = 23), respectively, of the studies. Smaller subsets of studies (~2%, n = 3 and 4) used either mixed engineering methods (e.g., OEX of one target with the KO of another target) or other engineering approaches.

Targeted pathways or cellular mechanisms

Engineering strategies target a broad range of cellular pathways and mechanisms to overcome productivity bottlenecks in CHO cells. These include manipulation of apoptotic and proliferative pathways to achieve high cell numbers and maintain high viability throughout the production process, metabolic reprogramming to improve energy levels or reduce toxic byproducts, enhancing messenger RNAs (mRNA) processing and translation, as well as improving protein folding, processing, transport and PTMs by engineering of endoplasmic reticulum (ER)/Golgi molecules or proteins being involved in the unfolded protein response (UPR). Manipulation of transcription factors (TFs) as well as epigenetic engineering also represent powerful strategies. While not being a targeted pathway/cellular mechanism, non-coding RNAs (ncRNAs) were grouped as a distinct category within this in-depth analysis due to their wide-ranging and often unclear mRNA targets, where affected pathways are diverse and partially unknown.

In this comprehensive dataset, approximately half of the reports targeted pathways characterized by a relatively narrow set of direct downstream effectors including (Figure 2(C)):

(1) Apoptosis or proliferation (n = 27) with targets involved in cell fate decisions including survival, apoptosis, cell cycle control and stress response (e.g., DNA damage, metabolic stress, oncogenic signals) as well as genomic stability.[11–37]
(2) Metabolism (n = 15) with targets involved in the energy, central carbon, and redox mechanisms, nutrient transport and utilization (e.g., sugar and amino acid uptake), as well as lactate, nitrogen and ammonia control.[38–52]
(3) UPR/ER/Golgi/PTMs (n = 10) targets to optimize protein folding, maturation and secretion, glycosylation as well as Golgi and ER processing and intracellular trafficking.[38, 53–61]
(4) Translation (n = 5) with targets of the translation machinery itself (e.g., initiation and elongation), ER-targeting and translocation as well as mRNA processing and modifications (e.g., splicing, stability, localization).[62–66]
(5) Others (n = 16) with targets involved in cellular processes like membrane and lipid homeostasis, DNA repair/genome stability, cell architecture and stress resilience.[67–82]

Additionally, this dataset included studies on single engineered targets with a broad regulatory influence, being capable of modulating hundreds to thousands of genes, such as TFs (n = 18), including transcriptional

luciferase are grouped under "other reporter." (B) Distribution of the target engineering type used to enhance CHO cell production. Engineering types are color-coded and single versus combined target engineering are indicated by pattern (solid vs. dotted). (C) Distribution of the cellular pathways or mechanisms targeted by the engineering approaches. Engineering approaches with a narrow set of direct downstream effectors (blue) as well as approaches with a broad regulatory influence on the transcriptional (purple) and translational (green) level are shown. Combinatorial approaches are depicted in orange. (D) Distribution of the subcellular localization of the engineering targets. (E) Distribution of the reports with respect to engineering setting of the target. (F) Distribution of the maximal fold improvement reported for either titer or qp, overall and across different time periods. In the violin plots, the bold black dotted line represents the median; the thin dotted lines indicate the 25th and 75th percentiles. The overall median improvement of 2.1-fold is shown as a bold gray dotted line. Statistical significance was assessed using the Kruskal-Wallis test. *(m)Ab- (monoclonal) antibody, combi – combined, EPO – erythropoietin, OEX- overexpression, KO – knockout, KD – knockdown, nc- non-coding, ns – not significant ($p \geq 0.05$), PTM- post-translational modifications, TF- transcription factor, UPR – unfolded protein response, ER- endoplasmatic reticulum, (m)AB- (monoclonal) antibody, GFP – green fluorescent protein, SEAP – secreted alkaline phosphatase, qp – specific productivity*.

regulators and stress-responsive transcription factors to improve CHO cell processes including the protein synthesis capacity and cellular adaptation to production stress;[83–100] and epigenetic modifiers ($n = 5$), including targets involved in DNA methylation, histone modification, and chromatin remodeling, for example, to indirectly enhance stable transgene expression as well as improve clonal stability in the production process and tune of cellular processes like secretion.[101–105]

A further prominent subset of this dataset includes reports on the engineering of ncRNAs ($n = 32$).[106–137] This group is dominated by microRNAs (miRNAs), which have garnered substantial interest since their first reports on analysis[138] and manipulation[126] in CHO cells, due to the fact that one miRNA usually regulates up to hundreds of different mRNAs and therefore can influence whole pathways or even more complex cellular mechanisms.

In many publications of this dataset, multiple targets from different pathways were engineered either separately or in combination ($n = 32$).[81, 139–169] In 4 reports the pathway of the target(s) was unknown.[170–173]

Subcellular localization of targets

In addition to the pathway, the subcellular localization of engineered targets provides insight into their functional role in improving CHO cell productivity. Nuclear targets are typically involved in gene regulation, epigenetic modification, or DNA replication, while mitochondrial targets can influence energy metabolism. ER and Golgi targets modulate protein folding, quality control, and trafficking, proteins in the cell membrane are often transporters, receptors or structural elements, while cytoplasmic targets are functionally diverse. In this dataset, about one-third of studies ($n = 55$) reported on cytoplasmic targets, while nuclear targets were addressed in one-fifth ($n = 29$) (Figure 2(D)). Roughly 10% focused on mitochondrial ($n = 17$) or ER/Golgi-localized proteins ($n = 13$) and 5% on cellular membrane proteins ($n = 8$). Another fifth of publications ($n = 38$) reported on target(s) with combinations/multiple subcellular localizations, while the small group of "other" target localizations includes, for example, reports on targets in additional cellular structures such as the cytoskeleton or unknown localization ($n = 4$).

Target gene origin and transient vs. stable target engineering

The species origin of the overexpressed gene can influence engineering outcomes through factors such as codon usage and molecular compatibility.[174] Among the 103 studies of this dataset using OEX, approximately half (51%) used CHO-derived target coding sequences (CDS), which are likely to support optimal expression and function. Human CDS were used in about 27% of cases, including three that used codon optimization for CHO expression. Mouse CDS (8%) and CDS from other organisms (8%), such as silkworm or viruses, were also represented (Supplementary Figure S2A).

Stable engineering remains the gold standard in industrial CHO production, mostly due to process robustness, costs, and regulatory requirements. Consistent with this, two thirds of the publications within this dataset reported on stable target engineering (65%, $n = 106$), while one fifth reported on transient (21%, $n = 35$) and the minority on both transient and stable target engineering (14%, $n = 23$), see Figure 2(E).

Author affiliation and number of publications per year

The interplay between academic and industrial publications in CHO cell engineering reflects a synergistic dynamic, where in many cases academia drives foundational discovery and tool development, while industry contributes translational insight and process optimization, increasingly converging through collaborative research that bridges innovation with biomanufacturing application. This can be seen within this dataset, where about half of the publications originated from academic institutions (48%), while 40% were coauthored by academic and industrial partners. A smaller proportion (12%) was authored solely by industry (Supplementary Figure S2B). A contributing factor to the lower numbers of industry-authored reports may be that exploratory work conducted in industrial settings is not always published.

The number of scientific papers published annually has increased over the past few decades, driven by factors such as the growth in global research funding, expansion of research institutions, and the emergence of new journals and digital platforms.[175] This broader trend can also be seen in this dataset. While only 7–12 reports were published annually between 2011 and 2013, this number increased to 18 and 17 reports in 2023 and 2024, respectively (Supplementary Figure S2C). The observed increase in CHO cell engineering

publications over time may additionally be attributed to the economic success of mAbs and their derivatives, which likely channeled more research funding into the field and enabled greater scientific output.

Improvement-fold reported and top 6 publications

CHO cell productivity is typically reported either as titer (e.g., g/L) or qp (e.g., pg/cell/day). However, absolute titer and qp levels depend on multiple factors, including but not limited to expression vector design, CHO host cell subtype, the production process, and media/feed strategies employed. To enable direct cross-study comparisons of the success of a given engineering approach, it was therefore most appropriate to focus on fold improvement of titer and/or qp. Moreover, due to substantial heterogeneity in data reporting across studies, where about one third of reports did not report initial titer or qp values and only 40% reported both, titer and qp (Supplementary Figure S2D), the derivation of absolute measures of engineering success was not universally possible. Across all 164 publications, the median improvement reported was 2.1-fold (Figure 2(F)). Most studies reported improvements between 1.6- and 3.4-fold (interquartile range), while only a few outliers exceeded 4-fold gains, with some reaching up to 35-fold. To investigate temporal trends, publications were grouped into 2–3 year intervals. Median improvement folds ranged from 1.8 to 2.5 across all time periods (2011–2024), with a slight decrease over time; however, no conclusive statement about this trend could be made (Kruskal-Wallis test, $p = 0.0773$). Positive outliers (> 10-fold) were reported throughout the timeframe studied. Interestingly, the temporal trend of maximal titers reported ($n = 112$) showed a different pattern: The median of the titers reported over time increased from 56 mg/L in 2011–13 to a maximum of 677 mg/L in 2020–22, with an overall median of 245 mg/L (Supplementary Figure S2E).

The six publications within this dataset reporting the highest improvement-fold illustrate the diversity of successful engineering strategies: 1) A 35-fold titer increase reached by deletion of the telomeric region of chromosome 8, thereby reaching final mAb titers of 4.5 g/L in a 14-day fed batch process.[171] Identified via functional transcriptomic analysis of high and low producers, follow-up investigations identified the KO of C12orf35 as being responsible for higher productivities and shorter recovery times.[170] 2) Artificial introduction of aneuploidy by 3-aminobenzamide, causing a 33-fold higher productivity with a final qp of 18 pg/cell/day,[68] which was based on preceding findings that high producing CHO clones often carry chromosomal aberrations. 3) Although not aiming for CHO production improvement but for increased sialylation of recombinant therapeutic proteins, a 30-fold increase in mAb productivity was observed by stable overexpression of the 2,6-sialyltransferase, ST6GAL1[61] without reporting a quantitative titer or qp. 4) Combinatorial engineering of a lactate dehydrogenase-A (LDH-A) KD and OEX of the anti-apoptotic protein Bcl-2, improving the production of a Fc-fusion protein 21-fold to a titer of 300 mg/L[148]. 5) Conditional expression of a cell-cycle checkpoint regulator, the mutant cell division cycle 25 homolog B (mCDC25B), improving production of a bispecific antibody (bsAb) 15-fold to a qp of 1.6 pg/cell/day.[11] 6) A 13.6-fold enhanced mAb production reached by co-overexpressing of the B lymphocyte-induced maturation protein-1 (BLIMP1) and the active form of the X-box binding protein 1 (XBP1s), reaching titers of 630 mg/L and a qp of 45 pg/cell/day.[98]

Target engineering methods

Trends over time

The manipulation of CHO cells to enhance recombinant protein production can be achieved through various strategies. OEX of endo- or exogenous genes has been commonly used since the late 1990s, facilitated by well-established cloning, transfection, and selections techniques. In contrast, KO was historically more complex and time-consuming prior to 2011. This was primarily due to two factors: 1) The CHO genome sequence was not publicly available until 2011,[8] complicating targeted genetic manipulations like KO; and 2) the KO-technologies available at the time were limited by the complexity of design of the DNA-binding protein subunit in order to recognize the particular DNA sequence of interest[176–178] and by the costs for access to the individual technologies. These include zinc-finger nucleases (ZFNs), which have been used in CHO cell engineering since 2008.[179] Additionally, transcription activator-like effector nucleases (TALENs) have been used in CHO cell engineering since 2013.[180] The landmark publication on the CRISPR-Cas9 system for genome editing in 2012,[181] allowing straight-forward KO of genes, revolutionized

the field of biotechnological engineering, and quickly led to successful applications of this technology in CHO cell engineering.[144,182] In addition to OEX and KO, KD has emerged as another prominent strategy, downregulating target expression. KD can be achieved through various mechanisms, including transcriptional repression using CRISPR interference, CRISPRi,[70] mRNA interference, RNAi by siRNAs causing mRNA degradation[183] or miRNAs mostly causing translational repression,[126] as well as using miRNA sponges to downregulate miRNAs.[184] A timeline of key milestones that have influenced CHO engineering is presented in Figure 3(A).

For this review, engineering mechanisms were defined based on the primary target. For instance, both protein-coding genes and miRNAs were categorized as OEX when the target expression was upregulated, despite the latter typically leading to downregulation of secondary target mRNA translation. In this dataset, approximately three quarters of publications from 2011 to 2016 reported on using OEX to enhance productivity (2011–13: 21 of 28; 2014–16: 22 of 30), Figure 3(B). From 2017 to 2019, this decreased to 50% (16 of 32) and since then the percentage of reports on OEX for CHO cell engineering has remained at a lower level, 68% in 2020–22 (26 of 38) and 53% in 2023–2024 (19 of 36), compared to the 2011–2016 period.

The number of reports on KD approaches remained relatively constant from 2011 to 2024, averaging about one publication per year and accounting for 10–25% of studies during each time period. The few publications on mixed strategies ($n = 3$) combined multiple engineering methods, such as KD of LDH to decrease lactate levels with Bcl2 OEX to enhance growth;[148] KD of the hydrolase HEX combined with OEX of the galactosaminyl transferases GALNT1 and C1Galt1 for an improved O-glycosylation and production;[53] and KO of the pro-apoptotic proteins apoptosis regulator bcl-2-like protein 4 (BAX) and Bcl-2 homologous antagonist/killer (BAK) combined with inducible OEX of the TFs cMYC and BLIMP1.[159] Within the last four years, "other" successful CHO engineering approaches were published, including artificial introduction of aneuploidy,[68] a hydrogen peroxide evolved cell line;[71] the introduction of beneficial mutations in genes such as ribosome RPL10-R98S;[64] and a sub-physiological temperature evolved cell line.[158]

The number of published KO approaches increased over time, with 2 reports (7%) in 2011–2013, 5 reports (17%) in 2014–2016, and 7–10 reports (18–25%) in each of the 2017–19, 2020–22 and 2023–24 periods. The increase in reports using the KO method over time is mainly attributed to the rise of CRISPR-Cas, as reflected in the shift of KO technologies applied (Figure 3(C)). From 2011 to 2013, both KO studies of this dataset used the ZFN strategy.[14,72] Between 2014 and 2016 two of five studies applied CRISPR-Cas,[46,144] while the majority of reports relied on alternative KO technologies, including TALENs,[170] ZFNs[73] and MTX-induced deletions.[171] By 2017–2019 CRISPR-Cas had become the dominant KO method, with seven of nine publications applying this strategy,[20,40,53,69,103,129,185] while two reports used TALEN and/or ZFN.[74,104] From 2020 through 2024 all KO studies in this dataset used CRISPR-Cas ($n = 7$ and 10).[1530183029–31,50,52,85,105,106,152,159,162,163,167,169] In addition to KO approaches, CRISPR technology has also enabled novel engineering target discovery strategies, including genome-wide activation screens of silenced loci, leading, for example, to the identification synaptonemal complex central element protein Syce3, enhancing bsAb production upon OEX.[161] The beneficial impact of CRISPR-Cas on CHO cell engineering is reflected in a broader trend toward an increasing emphasis on KO-based approaches, as well as expanded opportunities for engineering target screening and targeted genome editing.

Effect on the improvement fold achieved

To determine whether the engineering method affects the improvement fold, the full dataset of 466 engineered targets was compared in terms of the maximum improvement fold reported across five categories: OEX, KO, KD, Mix, and Other (Figure 3(D)). While the median improvements for OEX and KD (both 1.6-fold) matched the overall target median, the KO method yielded a higher median improvement (1.8-fold). Although comparing the five groups using the Kruskal-Wallis test suggested some evidence of difference between the groups ($p = 0.0293$), performing pairwise comparisons with correction for multiple testing using Dunn's approach did not allow clear conclusions for any of the group comparisons (all $p \geq 0.2432$). The three publications from the past five years reporting the highest improvement folds using KO approaches include KO of the epigenetic players activating transcription factor 7-interacting protein (ATF7IP) and histone methyltransferase SET domain bifurcated 1 (SETDB1) improving mAb production

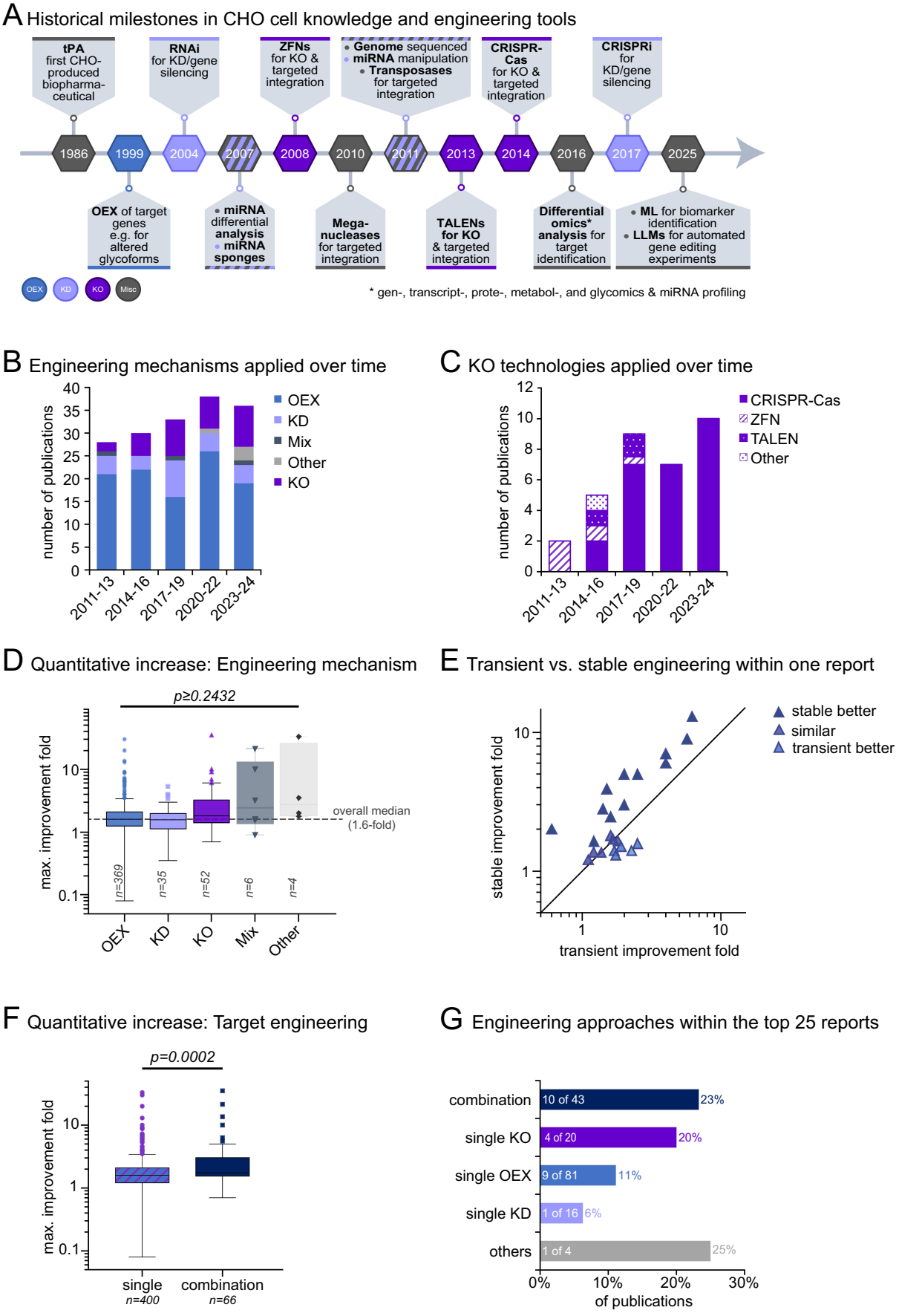

Figure 3. CHO cell target engineering methods and their impact on biopharmaceutical production. (A) Key milestones that have significantly influenced CHO cell engineering since 1986. (B) Number of publications across different time periods,

3.9-fold,[105] triple KO of the protein kinase R-like ER kinase (PERK) together with the pro-apoptotic proteins BAX and BAK improving mAb production 3.5-fold[152] and KO of the electron transport chain component NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 (Ndufa13) enhancing Fc-fusion protein production 3.1-fold.[50] Due to small sample sizes, results for the categories Mix and Other (n = 6 and 4, respectively) must be interpreted cautiously. Overall, while KO may be more effective in improving CHO productivity, this dataset does not allow for definitive conclusions.

In addition to the engineering method, the engineering setting – transient versus stable – is often discussed particularly in terms of whether transient outcomes predict those in stable systems, which are the industry standard. As discussed above and illustrated in Figure 2(E), the majority of publications within this dataset (65%, n = 106) report on stable target engineering. Of the 35 studies that exclusively used transient target engineering, over one third focused on screening and validation approaches, including differential expression analyses (proteins, mRNAs, miRNAs) comparing high- and low-producing CHO cells or CHO and human/plasma cells, as well as siRNA and mRNA mimic library screenings.[96,102,110,113,124,125,137,147,151,154,157,165,186] Within our dataset, 23 publications reported on both transient and stable target engineering. Comparing the maximum improvement folds achieved with both approaches in a paired analysis revealed that stable target engineering generally outperformed transient approaches (Wilcoxon matched-pairs test: p = 0.0094, data not shown). As shown in Figure 3(E), in 12 studies stable target engineering led to 0.4- to 6.8-fold greater improvements.[24,51,58,64,75,90,92,100,122,149,165,170] In six cases, both approaches produced similar results (≤0.2-fold difference).[25,82,97,133,136,150] In contrast, in five studies transient target engineering slightly outperformed the stable approach (0.3- to 0.9-fold).[53,108,116,117,123] It can therefore be concluded that while transient engineering is a valuable screening tool, stable expression typically yields higher or comparable productivity improvements.

Single versus combinatorial target engineering

As illustrated in Figure 2(B), the engineering methods (OEX, KD, KO) were used either for single or combinatorial target modification. Most studies (73%, n = 119) used single-target strategies, while 14% (n = 23) used combinatorial approaches and 13% (n = 22) applied both (Supplementary Figure S3A).

To assess differences in effectiveness, all 466 engineered targets of this dataset were analyzed (Figure 3(F)). Combinatorial engineering yielded a significantly higher median improvement (1.75-fold) compared to single-target strategies (1.6-fold; Mann Whitney test p = 0.0002). The three publications from the past five years reporting the highest improvement folds using combinatorial target engineering approaches include KO of the pro-apoptotic proteins BAX and BAK combined with inducible OEX of the TFs cMYC and BLIMP1/PRDM1 improving mAb production 10-fold,[159] combined OEX of gene-specific regulatory long ncRNAs (SINEUPs) and ubiquitous chromatin-opening elements (UCOEs) improving mAb production 5-fold,[149] and a context-specific mAb engineering approach (with 32 genes tested at varying expression levels and in different combinations), where the best results were achieved with combined OEX of the TF XBP1s and additional light chain OEX improving mAb production 3.5-fold.[157]

categorized by the target engineering mechanism reported: OEX, KD, KO, mixed and other strategies (Mix, Other). (C) Number of reports using specific KO technologies over 2–3 year time intervals. (D) Distribution of the maximal fold improvement reported for each of the 466 individual engineering targets, stratified by the type of engineering mechanism used. Statistical analysis was performed using the Kruskal-Wallis test (p = 0.0293) followed by Dunn's pairwise comparisons with correction for multiple testing (all $p \geq 0.2432$). (E) Comparison of improvement folds between transient and stable engineering within the same study. Reports in which stable engineering yielded >0.2-fold higher improvement are shown in dark blue; those with similar effects (≤0.2-fold difference) in purple; and those where transient engineering achieved better results (>0.2-fold difference) in light blue. (F) Distribution of maximal fold improvements across all targets (n = 466), comparing single versus combinatorial engineering. Statistical analysis was performed using the Mann-Whitney test. (G) Distribution of the top 25 publications reporting ≥5-fold improvement, grouped by target engineering method and normalized to the total number of publications reporting each respective method. *KD – knockdown, KO – knockout, LLM – large language model, miRNA – micro ribonucleic acid, Misc – miscellaneous, ML – machine learning, OEX – overexpression, RNAi – mRNA interference, siRNA – small interfering ribonucleic acid, TALEN – transcription activator-like effector nuclease, tPA – tissue-type plasminogen activator, ZNF – zinc finger nuclease.*

Among the top 25 studies of this dataset reporting ≥5-fold improvement,[11,14,19,21,24,61,65,68,69,73,75,92,98–101,104,142,146,148,149,155,159,170,171] combinatorial strategies were also prominently represented (40%, $n = 10$; Supplementary Figure S3B). When normalizing the number of top-performing studies by the total number of publications using each respective engineering strategy, combinatorial and single KO approaches showed the highest success rates –23% and 20%, respectively – compared to single OEX (11%) and single KD (6%) (Figure 3(G)). "Other" engineering methods also ranked very well (25% success rate), though this result should be interpreted cautiously due to the small sample size in this group ($n = 4$). These findings may suggest that combinatorial approaches are more likely to yield high improvement folds, and KO strategies could be particularly effective in driving substantial gains in CHO cell production.

Engineering targets

Trends over time

With the publication of the CHO K1 genome sequence in 2011 and the rapid development of supporting technologies – such as high-throughput omics and computational modeling[7,187] - new avenues for CHO cell engineering were opened. To evaluate whether these advances influenced the breadth of target screening, the number of engineering targets evaluated per publication was examined over time. As shown in Figure 4 (A), there is a clear upward trend: From a mean of 1.75 targets per publication in 2011–2013, this number rose to a peak of 4.5 targets in 2020–2022 and remained above two in 2023–2024 (mean: 2.9). Examples of omics-based approaches and target validation include transcriptomic profiling of DTE- and aggregation-prone bsAbs;[147] high and low producer comparison in respect to miRNAs[114,137] and the transcriptome;[143,166] as well as multi-omics comparisons between CHO and plasma cells.[113,165]

Analyzing the improvement for each of the 466 targets within this dataset revealed a median of 1.6-fold (Figure 4(B)), which is lower than the median improvement per publication (2.1-fold, see above and Figure 2(F)). This difference is expected, as the publication-level analysis focused on the highest performing target. Furthermore, it could be observed that the median improvement fold decreased from 2.1 in 2011–2013 to 1.5 in 2020–2022, coinciding with a substantial increase in the number of reported targets (from $n = 49$ to $n = 172$). These findings suggest that broader target evaluation over time has included more modestly performing candidates, thereby lowering the median improvement.

Targets published in multiple reports

To identify promising engineering targets, the recurrence of individual targets reported across the 164 publications was analyzed. Among the 466 entries in the dataset, 370 distinct targets were reported. The majority were described in only one publication ($n = 321$; 87%), while a smaller subset appeared in multiple reports ($n = 49$; 13%) (Figure 4(C)). Among the 17 most frequently reported targets, six TFs (XBP1s, Blimp1, ATF6c, ATF4, CHOP, and MYC), three apoptosis/proliferation regulators (BAK, BAX, and Bcl-xL), four ER/Golgi/UPR proteins (BIP, PDI, CypB and PDIA4), three ncRNAs (miR-17, miR-7 and miR-92a), and one metabolic target (LDH) were identified, depicted in Figure 4(D).[14–17,26,28–30,33–35,37,42,83–86,91,93,95,98,99,110–112,126–128,130,139–142,144,147,148,152,154–156,159,160,162,185]

To assess consistency in outcomes, focus was set on XBP1s, BAX, BAK, and Bcl-xL – the most frequently reported targets in this dataset (Table 1). Although direct comparisons are limited by differences in expression settings and systems, recombinant protein types, and reporting formats, three patterns emerged. First, single target OEX of XBP1s or Bcl-xL typically yielded improvements ranging from 1.0- to 4-fold, while combinatorial engineering involving XBP1s led to enhancements up to 13.6-fold. Second, improvements were reported across a range of products, including mAbs, (DTE) Fc-fusion proteins, and other therapeutic proteins such as EPO, while mAbs dominated and showed the highest gains. Third, engineering of the apoptosis regulator BAX via KD or KO was consistently performed in combination with KD or KO of BAK, and vice versa, with reported improvement between 1.3- and 10-fold. Notably, in 8 of the 10 studies, additional targets were co-engineered. In summary, even for identical targets, improvement folds varied across studies, for example, due to differences in production and target engineering contexts. Nonetheless, combinatorial approaches consistently outperformed single-target strategies.

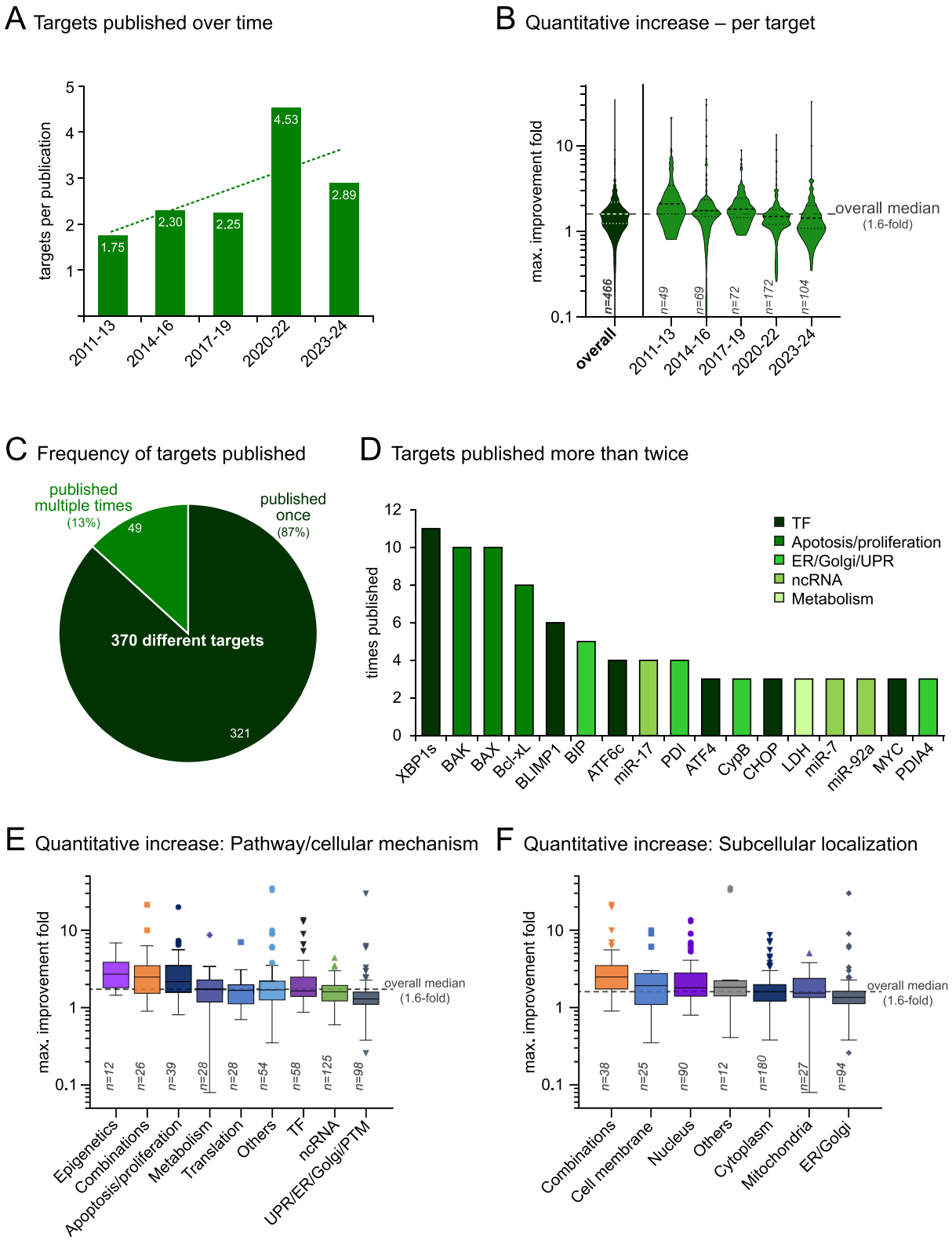

Figure 4. Characteristics and performance of engineering targets. . (A) Average number of targets reported per publication, shown as mean values across 2–3 year time periods. A trend toward an increasing number of reported targets over time is indicated by the dotted green line. (B) Distribution of the maximal fold improvement in either titer or qp reported for each target, overall and by time period. In the violin plots, the bold dotted line represents the median, while thin dotted lines indicate the 25th and 75th percentiles. (C) Distribution of engineering targets reported in only one publication versus those reported in multiple publications. (D) Number of publications per target for all targets reported in more than two publications. (E) Distribution of the maximal fold improvement according to cellular pathway or mechanism targeted by the engineering approach. (F) Distribution of the maximal fold improvement by subcellular localization of the engineered target. *ER- endoplasmatic reticulum, nc- non-coding, PTM- post-translational modifications, TF- transcription factor, UPR- unfolded protein response.*

Table 1. Extracted key data of the 4 most often published targets within this dataset.

Engineered target(s)	Max. improvement fold**	Initial titer/ qp class*	Engineering mechanism	Recombinant protein production	Target engineering	Re-combinant protein produced	Engineering target origin	Targeted pathway(s)	Main cellular localization target	Author affiliation	Reference
XBP1s	1.37	low - titer	OEX	transient	transient	mAb	murine	TF	Nucleus	Academic	93
XBP1s & ERO1-La	6.3	low - titer	COMB, OEX	transient	stable	mAb	human	COMB (TF, PTM)	Multiple (Nucleus, ER/Golgi)	Mix	155
XBP1s	1	NA	OEX	stable	stable	tPA	human	TF	Nucleus	Academic	141
XBP1s	4	NA	OEX	stable	stable	mAb	human	TF	Nucleus	Academic	91
XBP1s	1.86	low - titer & qp	OEX	transient	transient	mAb	human	COMB (TF, UPR/ ER/Golgi)	Multiple (Nucleus, ER)	Mix	140
XBP1s	1.48	NA	OEX	transient	transient	Fc fusion protein, DTE	human	TF	Nucleus	Mix	142
XBP1s	1.8	low - titer	OEX	transient	transient	mAb	CHO	TF	Nucleus	Industry	156
XBP1s & LC	3.54	NA	COMB, OEX	transient	transient	mAb	CHO & human	COMB (TF, LC)	Multiple (Nucleus, LC)	Mix	157
XBP1s & BLIMP1	13.6	low - titer & qp	COMB, OEX	stable	stable	mAb	human	TF	Nucleus	Academic	98
XBP1s & MYC	2.5	low - titer & qp	COMB, OEX	stable	stable	EPO	human	TF	Nucleus	Academic	86
XBP1s & PDI	2.37	low - titer & qp	COMB, OEX	transient	transient	mAb	unclear	COMB (TF, UPR/ ER/Golgi)	Multiple (Nucleus, ER)	Academic	160
Bcl-xL	1.87	low - titer	OEX	stable	stable	EPO	CHO	Apoptosis	Mitochondrial membrane	Academic	34
Bcl-xl	1.16	low - titer & qp	OEX	stable	stable	EPO	unclear	Apoptosis	Mitochondrial membrane	Academic	28
Bcl-xL	3.5	low -titer	OEX	transient	stable	mAb	unclear	Apoptosis	Mitochondrial membrane	Academic	26
Bcl-xL	2.4	low - titer	OEX	transient	transient	Fc fusion protein	human	Apoptosis	Mitochondrial membrane	Academic	35
Bcl-xL	1.4	low - titer	OEX	stable	stable	Fc fusion protein	CHO	Apoptosis	Mitochondrial membrane	Academic	37
Bcl-xL	2.1	low - titer	OEX	stable	transient	mAb	mouse	Apoptosis	Mitochondrial membrane	Mix	16
Bcl-xL	1.8	low - titer	OEX	transient	transient	mAb	human	Apoptosis	Mitochondrial membrane	Mix	33
Bcl-xL	2.7	low - titer	OEX	transient	transient	mAb	human	Apoptosis	Mitochondrial membrane	Mix	17
BAK & BAX	5	NA	COMB, KO	transient, TGE	stable	mAb	NA	Apoptosis	Mitochondrial membrane	Industry	14
BAK, BAX & FUT8	2.2	low- qp & titer	COMB, KO	transient	stable	mAb	NA	COMB (Apoptosis, PTM)	Multiple (Mitochondrial membrane, Golgi)	Mix	144
BAK, BAX & Neu 1, 2, 3	1.4	low - titer	COMB, KO	stable	stable	EPO	NA	COMB (Apoptosis, PTM)	Multiple (Mitochondrial membrane, secreted)	Mix	167
BAK, BAX & PDK1	1.42	high - titer	COMB, KD	stable	stable	mAb	NA	COMB (Apoptosis, Metabolism)	Mitochondrial membrane	Mix	139
BAK, BAX & BOK	1.54	low - qp	COMB, KO	stable	stable	mAb	NA	Apoptosis	Mitochondrial membrane	Mix	29
BAK & BAX	1.5	high - titer & qp	COMB, KO	stable	stable	mAb	NA	Apoptosis	Mitochondrial membrane	Industry	15
BAK, BAX & PERK	3.5	high - titer	COMB, KO	stable	stable	mAb	NA	COMB (Apoptosis, UPR/ER/Golgi)	Multiple (Mitochondrial & ER membrane)	Industry	152
BAK, BAX & BCKDHa/b	1.5	NA	COMB, KO	stable	stable	mAb	NA	COMB (Apoptosis, Metabolism)	Mitochondrial membrane	Industry	162
BAX, BAK, BOK	1.3	high- qp	COMB, KO	stable	stable	mAb	NA	Apoptosis	Mitochondrial membrane	Mix	30
BAK, BAX, BLIMP	10	low - titer & qp	COMB, KO & OEX	stable	stable	mAb	NA	COMB (Apoptosis, TF)	Multiple (Mitochondrial membrane; nucleus)	Mix	159

*High titer/qp: ≥1 g/L and/or 10 pg/cell/day.
Low titer/qp: <1 g/L and/or 10 pg/cell/day.
NA: No quantitative titer/qp reported or titer/qp in different units (GFP, luciferase, SEAP). **Caused by XBP1s, or Bcl-xL, or BAX & BAK.
EPO – erythropoietin, ER – endoplasmatic reticulum, COMB – combination, KD – knockdown, KO – knockout, (m)Ab – (monoclonal) antibody, LC – light chain, NA – not applicable, OEX - overexpression, TF – transcription factor, tPA – tissue-type plasminogen activator, UPR – unfolded protein response.

Targeted pathway/cellular mechanism

Given the range of pathways and cellular mechanisms targeted in CHO cell engineering (Figure 2 (C)), we compared their relative effectiveness in improving CHO cell production. Analysis of 466 targets revealed four trends (Figure 4(E)). First, engineering efforts focusing on epigenetics, pathway combinations, and apoptosis/proliferation yielded the highest median improvements (2.7-, 2.5-, and 2.2-fold, respectively), clearly above the overall target median of 1.6-fold. The up to 3 publications of each group with the highest improvement fold reported within the last 5 years are as follows: 1) Epigenetics: As mentioned above, KO of ATF7IP and SETDB1,[105] and combinatorial KD of DNA methyltransferases DNMT1 and 3a and DNA demethylating proteins TET2 and 3 improving EPO-Fc production 1.5-fold;[102] 2) Pathway combinations: As mentioned above, triple KO of PERK, BAX and BAK,[152] OEX of SINEUP and UCOE,[149] and KO of BAX and BAK combined with inducible OEX of cMYC and BLIMP1;[159] and 3) Apoptosis/proliferation: OEX of Sirtuin 6 (SIRT6), a stress responsive protein deacetylase and mono-ADP ribosyltransferase improving mAb production 2.8-fold,[27] KO of the apoptotic protease activating factor 1 (APAF1) improving IL-3 production 2.2-fold,[31] OEX of mutated phosphatase and tensin homolog PTEN, a regulator of proliferation, improving mAb production 1.8-fold.[25] Second, targets related to metabolism, "others", translation, TFs, and ncRNAs achieved improvements near the overall median (1.6- to 1.7-fold). Third, UPR/ER/Golgi/PTM-related targets had the lowest median improvement at 1.3-fold. Fourth, positive outliers (≥10-fold improvement) could be found in publications reporting on engineering of combinations, apoptosis/proliferation, "others", TFs, and UPR/ER/Golgi/PTMs. These include deletion of the telomeric region of chromosome 8 ("others"),[171] introduction of aneuploidy ("others"),[68] ST6GAL1 OEX (PTM),[61] LDH-A KD combined with BCL-2 OEX (combination),[148] mCDC25B OEX (proliferation),[11] BLIMP1 and XBP1s co-OEX (TF),[98] and BAX/BAK KO together with OEX of cMYC and BLIMP1 (combination),[159] as well as OEX of the TF yin yang 1 (YY1) improving mAb expression 13-fold,[92] and KD/KO of the IGF1 receptor improving human IGF-1 production 10-fold ("others").[73] In summary, targeting apoptosis/proliferation, epigenetic mechanisms, and pathway combinations could potentially be more effective strategies for improving recombinant protein production in CHO cells.

Cellular localization of the target

As with the pathways, we investigated whether the subcellular localization of engineered targets (as described above and Figure 2(D)) influenced the degree of productivity improvement. Analysis of the full dataset ($n = 466$) revealed four main findings (Figure 4(F)): First, targets combined from different subcellular localizations, and those localized in the cell membrane, the nucleus, or other cellular locations achieved median improvements above the overall median of 1.6-fold (2.5-, 1.9-, 1.8-, and 1.8-fold, respectively). The up to 3 publications of each group with the highest improvement fold reported within the last 5 years include: 1) Different subcellular localizations: As mentioned above, OEX of SINEUP and UCOE,[149] BAX/BAK KO together with OEX of cMYC and BLIMP1,[159] and OEX of XBP1s together with additional light chain OEX[157]; 2) Cell membrane: OEX of membrane located tumor necrosis factor receptor superfamily member 8 (TNFRSF8) – identified by a CRISPR activation screening – improving bsAb production up to 2.5-fold,[161] and OEX of transferrin receptor protein (TFRC) – identified among others via multi-omics plasma and CHO cell comparison – improving mAb production 1.2-fold;[165] 3) Nucleus: As mentioned above, BLIMP1 and XBP1s co-OEX,[98] and OEX of BLIMP1;[68,83,99,100] and; 4) Other cellular locations: OEX of the cytoskeletal protein alpha cardiac actin (ACTC1) improving mAb production 2-fold.[67] Second, cytoplasmic and mitochondrial targets aligned with the overall median (1.6- and 1.5-fold, respectively). Third, ER/Golgi targets yielded lower median improvements (1.4-fold), similar as reported above for the pathways. Fourth, positive outliers (> 10-fold improvement) were reported for the combination of targets from different subcellular localizations, membrane-associated, nuclear, other and ER/Golgi localized targets. Thus, targets localized in the nucleus and the cell membrane, as well as combinations of targets from multiple subcellular localizations, could potentially be particularly promising routes for enhancing CHO cell production performance.

Recombinant production

Type of recombinant protein produced and transient vs. stable recombinant production

A frequently debated question in CHO cell engineering is whether the type of recombinant protein influences the maximum achievable titer and the success of engineering strategies, given differences in structural complexity and folding requirements. To address this, production improvements were analyzed for each protein class described in Figure 2(A) (mAbs, antibody derivatives, other therapeutic proteins, and other reporters). Although comparing the four groups using the Kruskal-Wallis test suggested some evidence of difference between the groups ($p = 0.0458$), performing pairwise comparisons with correction for multiple testing using Dunn's approach, did not allow clear conclusions for any of the group comparisons (all p-values ≥0.2324), see Figure 5(A). Nevertheless, it is worth highlighting that CHO cell engineering for antibody derivatives was particularly successful, with a median improvement of 2.7-fold, which was higher than the overall median and the improvement of mAb production (2.1-fold). The three publications with the highest improvement fold reported for antibody derivative production engineering within the last 5 years include, beside the above-mentioned KO of Ndufa13,[50] OEX of the constitutively active TF variant of the cAMP response element binding protein VP16-CREB improving etanercept expression 3.9-fold[90] and a hydrogen peroxide evolved cell line with upregulated antioxidant defense genes improving DTE bsAb production 3.5-fold.[71]

All currently marketed CHO-derived biopharmaceuticals are produced using stable single-cell clones. Accordingly, the majority of publications in this dataset (77%) reported results from stable recombinant protein expression, while 15% used transient production systems, and 8% used both (Supplementary Figure S4A). It can therefore be concluded that: 1) Within this dataset there was insufficient evidence to assert an association in the improvement fold achieved among different recombinant protein classes; 2) antibody-derivative production can be successfully enhanced through CHO cell engineering; and 3) most studies of our dataset report on stable recombinant protein production.

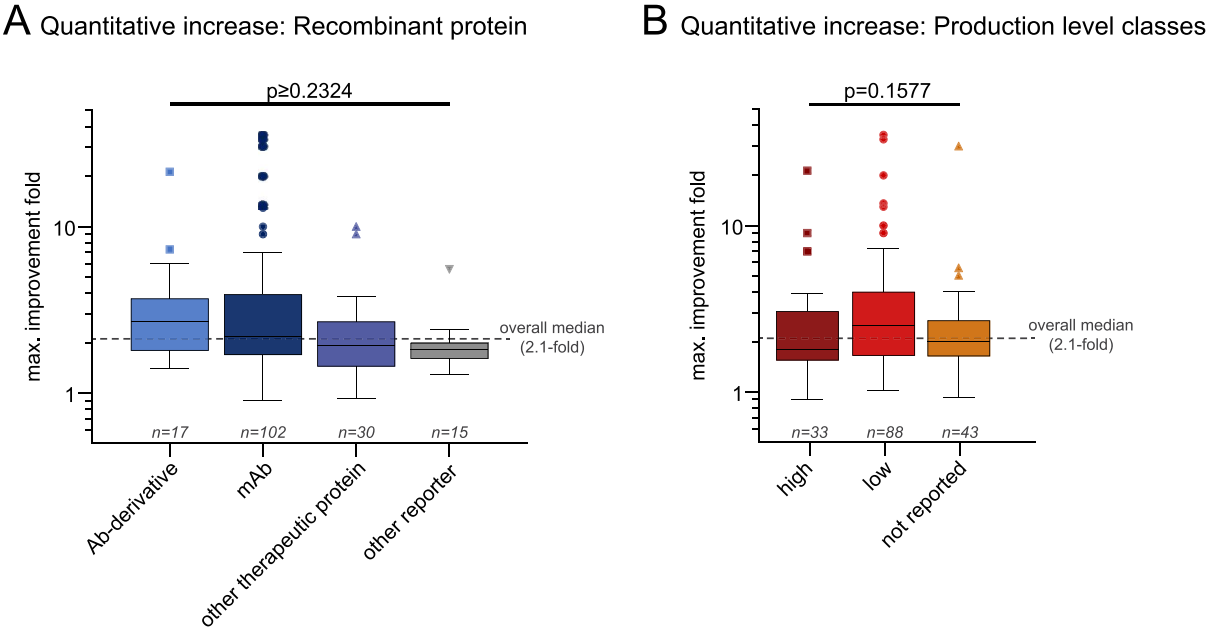

Figure 5. Influence of recombinant protein type and production level on reported improvement folds. (A) Distribution of the maximal fold improvement reported per publication, categorized by the type of recombinant protein produced. The "Ab-derivative" group includes Fc-fusion proteins, bispecific antibodies and single chain diabodies. "Other therapeutic protein" encompasses products such as blood factors (e.g., EPO), growth factors, and enzymes. "Other reporter" includes classical research reporter proteins such as GFP, SEAP or luciferase. Statistical analysis was performed using the Kruskal-Wallis test ($p = 0.0458$), followed by Dunn's pairwise comparisons with correction for multiple testing (all p-values ≥0.2324). (B) Distribution of the maximal fold improvement reported per publication by production classes: High initial production (titer ≥1 g/L and/or qp ≥ 10 pg/cell/day), low initial production (titer < 1 g/L and/or qp < 10 pg/cell/day) and "not reported" (no quantitative data on titer or qp provided). Statistical analysis was performed using the Kruskal-Wallis test ($p = 0.1577$). *(m)Ab - (monoclonal) antibody*.

Improving high versus low producing CHO cells

Another widely debated issue in CHO engineering is whether productivity improvements achieved in low-producing cells are transferable to high-producers, which may face different bottlenecks. To investigate this, the studies were categorized based on reported initial titers and/or qp: "high" (≥1 g/L and/or ≥10 pg/cell/day), "low" (< 1 g/L and/or < 10 pg/cell/day), and "not reported" (only improvement fold provided, no absolute titer or qp values). About one-fifth of the studies (20%, $n = 33$) engineered high producers, roughly half of the reports (54%, $n = 88$) used low-producing cells, and about a quarter (26%, $n = 43$) did not report initial titers or qp values (Supplementary Figure S4B). Comparison of improvement folds achieved among these groups showed that there was insufficient evidence to detect a statistical difference (Kruskal-Wallis test, $p = 0.1577$, Figure 5(B)). The median improvement for studies involving high producers was with 1.8-fold lower than the overall median (2.1-fold). The three publications reporting the highest improvement folds within the past five years are OEX of the TF BLIMP1 in combination with sodium butyrate improving mAb titer from 4.8 to 9.8 g/L and qp from 20 to 180 pg/cell/day,[100] combined OEX of the translational players ribosome-binding protein RRBP1/p180 and splicing factor subunit SF3b4 improving mAb titer from 3 to 5 g/L and qp from 8.3 to 34 pg/cell/day,[63] and KO of the electron transport chain component Ndufa13 improving Fc fusion protein titer from 1.2 to 2.75 g/L and qp from 7 to 21 pg/cell/day.[50] The median of low producer engineering was 2.5-fold, which is higher than the overall median. The 3 publications with the highest improvement fold reported within the last 5 years are artificially introduced aneuploidy improving bsAb productivity from 0.0075 to 0.25 pg/cell/day,[68] combined OEX of the TFs BLIMP1 and XBP1s improving mAb titer from 0.13 to 0.58 g/L and qp from 3.3 to 45 pg/cell/day,[98] and BAX and BAK KO combined with inducible OEX of cMYC and BLIMP1 improving mAb titer from 0.16 to 0.32 g/L and qp from 1.5 to 15 pg/cell/day.[159] Publications not providing an absolute titer or qp showed a median improvement of 2.0-fold. The 3 publications with the highest improvement fold reported within the last 5 years are a hydrogen peroxide evolved cell line improving bsAb titer 3.5- and qp 1.6-fold,[71] OEX of XBP1s together with additional light chain OEX improving mAb titer 3.1- and qp 3.5-fold,[157] and OEX of the mutated TF yes-associated protein 1 (YAP-1, YAP5SA) improving EPO titer 3.2- and qp 1.6-fold.[94]

To assess whether the analyses based on the maximal improvement fold were influenced by the initial titer or qp, we additionally examined overall and subgroup-specific comparisons of these metrics. When plotting initial titer and qp against the maximal improvement fold achieved, no clear trend emerged that would suggest low-producing cell lines yield higher improvement folds (Supplementary Figure S5A, B). A similar pattern was observed for the subgroups stratified by targeted pathway or cellular mechanism (Supplementary Figure S5C). For the subgroups combinations, apoptosis/proliferation and others, the data were distributed across the full range of initial titers (0.004 to 5500 mg/L). Subgroups that did not cover the full range included epigenetics (titer range 0.3 to 373 mg/L), metabolism (titer range 4.6 to 5500 mg/L), translation (titer range 10 to 3000 mg/L), TF (titer range 1.7 to 5500 mg/L) and ncRNAs (titer range 0.2 to 2400 mg/L); none of these subgroups showed a tendency toward studies reporting low initial titers, but rather medium to high titers. This indicates that the higher median improvement folds reported for engineering strategies such as epigenetics, apoptosis/ proliferation, and combinatorial approaches are most likely not simply driven by lower initial titers. In summary, although the data suggest a trend toward greater improvements in low-producing CHO cells, there was insufficient evidence within this dataset to confirm that low producers are inherently easier to engineer for productivity gains than high producers.

Discussion and outlook

In this review, we compiled and analyzed publications on CHO cell engineering approaches with effects on recombinant protein production since 2011. From 164 identified reports, key performance data of 466 engineered targets were extracted, resulting in a comprehensive key performance dataset that enabled trend analysis and the identification of critical factors, providing guidance for future CHO cell engineering approaches. While systematic literature reviews and in-depth analyses are well established in clinical research, such approaches remain rare in the bioprocessing field. A notable exception is the study by Golabgir et al., which focused on CHO production characteristics.[187] Published in 2016, their analysis was based on publications extracted exclusively from Web of Science using a title and abstract search and involved data extraction from 74 articles. However, the scope of their analysis was distinct from our study.

Their focus was on CHO phenotypes, culture performance, and production characteristics to identify differences between cell lines and correlations among variables such as growth rate, titer, and viability, rather than on target engineering and productivity improvement folds.

The strength of systematic reviews, such as the one presented here, lies in the structured and comprehensive literature search (covering the three most relevant databases) using clearly defined search terms and selection of publications based on predefined inclusion and exclusion criteria to ensure the identification of all relevant studies. Limitations of our systematic review include the restriction to reports published after January 2011, which was implemented for feasibility reasons and therefore excludes earlier contributions to CHO cell engineering. Additionally, as our study focused specifically on CHO cell engineering approaches, studies on process and media/feed strategy optimization, which have been major drivers of enhanced production levels, were not included. Generating datasets that integrate both engineering and process-related factors across a broader temporal scope would be highly valuable as a complementary follow-up study and may become increasingly feasible with advances in LLM-based data extraction.

It is possible that relevant studies were missed due to differences in database indexing or the use of nonstandard terminology by authors, and the restriction to English-language publications may have introduced additional bias. As with all literature-based syntheses, publication bias remains a further limitation, as studies reporting large improvements are more likely to be published than those with negative or marginal outcomes. Moreover, highly successful but patent-relevant findings from industrial research may not be published at all. Nevertheless, the reports on CHO cell engineering accumulated here provide key insights and underscore the need for more systematic review approaches in bioprocessing to guide future study designs. Furthermore, by extracting a consistent set of key process parameters from the selected literature, this review aggregated a large-scale dataset across studies, enabling conclusions that are not evident from individual publications.

The identified reports demonstrated a marked increase in the use of gene KO in CHO cell engineering since 2015, primarily driven by the adoption of CRISPR-Cas technologies (Figure 3(B,C)). This shift represents an important development, as OEX of engineering targets, particularly when combined with recombinant protein production, can impose a significant metabolic burden on the host cell, potentially limiting productivity gains.[188] This hypothesis is supported by the finding that KO-based approaches achieved a higher median improvement (1.8-fold) than OEX-based strategies (1.6-fold), although there was insufficient evidence to detect a statistically significant difference. Nevertheless, it is notable that in 2023 and 2024, the majority of reports (>50%) in our dataset still focused on OEX for CHO cell engineering, with only about one quarter reporting on KO approaches. Several factors may contribute to this imbalance, including the relative ease of applying more traditional OEX technologies, delays in the broader adoption of state-of-the-art methods, strategic preferences for adding new functions rather than removing limiting ones, and constraints of the current intellectual property KO technology environment.

The CRISPR-Cas landscape in particular is highly complex and competitive. Initial patents for its application in eukaryotic systems were filed in 2012, and with ongoing litigation, most patents are not expected to expire before 2032.[189] As a result, CRISPR-Cas is currently used in the industrial biopharmaceutical sector primarily for exploratory research rather than in production cell lines.[190] However, this is expected to evolve over the coming decade, as the technology holds promise not only for the generation of highly productive cell lines for biopharmaceutical manufacturing but also for enabling novel therapeutic options, such as gene therapies for genetic disorders.

Most of the studies in this dataset reported on stable recombinant protein production (Supplementary Figure S4A) as well as stable target engineering (Figure 2(E)), which aligns with current industry practices that rely exclusively on stable single-cell clones for safety and regulatory reasons. However, the COVID-19 pandemic underscored the urgent need for rapid therapeutic development, renewing interest in optimizing transient CHO expression systems.[81,191] Among the 25 publications in this dataset reporting exclusively on transient production, more than half ($n = 14$) specifically aimed at accelerating production timelines. With the potential to produce biologics weeks to months faster, transient expression has been applied in the rapid production of vaccine antigens for coronavirus treatment.[192] Despite its advantages, broader adoption of transient expression in industrial settings is currently limited by regulatory hurdles related to process robustness and reproducibility, comparatively lower productivity, and challenges in ensuring scalability and consistent product quality. An alternative strategy for accelerating development timelines involves

(semi-)targeted rather than random transgene integration.[193] Site-specific genomic integration using transposase-, recombinase-, or Cas9 nuclease-mediated systems can significantly reduce the time required to generate stable pools and single-cell clones. When paired with regulatory acceptance of stable pool-based manufacturing, this approach enables the initiation of Phase 1 clinical trials as early as six months after transfection.[194,195] Additional strategies aiming to speed up biopharmaceutical development include the incorporation of artificial intelligence (AI) and machine learning, as discussed in more detail below,[196,197] the use of stable cell pools instead of single cell clones,[198] as well as the implementation of automation, miniaturization, and high-throughput systems to enable faster processing and screening.[199]

This analysis revealed that combinatorial target engineering outperformed single target approaches in median improvement folds achieved across the overall dataset, as well as within specific pathways/cellular mechanisms and subcellular localizations of the targets (Figures 3(F,G) and 4(E,F)). Engineering targets located in the nucleus including epigenetic regulators, and those involved in apoptotic/proliferative pathways resulted in a higher median improvement fold. The success of apoptotic and proliferative targets is likely attributable to their direct impact on maintaining high cell densities and sustained viability during production. In the case of nuclear targets, their broad regulatory influence, often affecting hundreds to thousands of downstream genes, may underlie their effectiveness, producing outcomes that resemble those of combinatorial engineering strategies. Nevertheless, it should be recognized that epigenetic and TF engineering are more likely than single-gene modifications to produce unforeseen off-target effects, including increased risks of cell line instability.[200] To mitigate these risks, future CHO cell engineering efforts should incorporate rigorous cell line monitoring and leverage data-driven prediction tools to anticipate and manage potential adverse outcomes. Taken together, these observations support the notion that single gene or RNA engineering can improve cell numbers and viability, but has overall limited potential, and that greater productivity gains are achievable through the simultaneous targeting of multiple genes, RNAs, or pathways. This conclusion aligns with previous findings highlighting the multifactorial nature of bottlenecks in CHO cell production[92,143,201] and it provides a rationale for multi-targeted engineering approaches in future CHO optimization efforts.

Additionally, a clear increase in the number of engineering targets evaluated per publication over time (2011 – 2024) was observed. This trend reflects advancements in omics technologies that enable identification of candidate targets, and in high-throughput screening platform development facilitating their validation. These developments are accompanied by a significant expansion of knowledge, providing a strong foundation for more systematic and effective CHO cell engineering in the future.

However, a major challenge remains: The heterogeneity in reporting research findings. As previously noted by Golabgir et al.,[187] the absence of standardized data formats, particularly with respect to CHO cell lines, recombinant products, and media and culture conditions, hampers the comparability and reproducibility of data and processes, as well as reuse of data in comprehensive cross-study analyses like this one. This heterogeneity is further compounded by the frequent omission of numerical values reported for titer and qp, making cross-study comparisons even more difficult. This limitation is exemplified by the comparison of key performance data for the four most frequently reported targets in this dataset, XBP1s, BAK, BAX, and Bcl-xL. Although improvements in the expression of various recombinant proteins (e.g., mAbs, Fc-fusion proteins, blood factors) were consistently reported across both stable and transient expression systems, the observed improvement folds varied considerably between studies. These discrepancies are consistent with previous findings and can be attributed to factors such as differences in target expression levels, the species origin of the coding sequences, protein-specific production characteristics, as well as the production process and media used.[157,202,203]

Although our cross-study analyses provide a structured and quantitative overview of CHO cell engineering strategies, several methodological constraints rooted in the available literature must be acknowledged. The analyses conducted were univariate, assessing individual variables such as engineering method, targeted pathway, or cellular localization independently. Multivariate modeling, necessary to quantify interactions between factors and identify joint predictors of productivity, was not feasible. This limitation arises from several statistical challenges inherent to the dataset: Substantial heterogeneity in reporting and non-overlapping variable sets across studies violate key assumptions for multivariate approaches, including missing data, inconsistent output reporting, and consequently inadequate complete sample-to-variable ratios. A related limitation concerns the metrics used to quantify engineering success. Ideally, initial and

final titers or specific productivities would serve as absolute measures enabling robust cross-study comparisons. However, these values were not consistently reported. In one third of the identified publications, only relative changes or normalized values were provided. To enable quantitative integration of all studies, the improvement fold (based on titer or qp) was therefore used as the harmonized metric. While this approach allowed systematic comparison, it inevitably obscures baseline performance differences and limits the interpretability of absolute productivity gains. As a result, the findings of this review should be interpreted as identifying broad associations and trends rather than establishing causal relationships or predictive models. Nonetheless, the structured dataset created here forms an important foundation for future multivariate and machine-learning analyses as reporting becomes more standardized and larger, more complete datasets emerge.

Within the results reported above, we provide the three publications from the past five years reporting the highest improvement folds for each topic. This reporting on the positive outliers should be seen as providing some examples of success, while not aiming to provide suggestions for future engineering ideas and approaches.

As long as both academic and industrial research continue to use and report diverse experimental settings using variable metrics, the ability to compare results and draw meaningful conclusions about translatability will remain limited. One potential solution is to make use of the recently published NIST CHO cell line, developed by the U.S. National Institute of Standards and Technology. Designed as a well-characterized reference cell line, this stable mAb-producing clone (titer > 2 g/L) enables more consistent comparisons across studies. The associated fed-batch process, based on commercially available culture media, has also been externally validated.[204] To further facilitate efficient and effective comparisons in CHO cell engineering, additional standardization is needed. This includes the establishment of nomenclature conventions for reference cell lines;[205] and requirements by journals for key experimental data to be submitted in structured, machine-readable formats (e.g., excel files), enabling easier data extraction and reuse.

Uniform reporting based on a common reference cell line, combined with accumulated high-throughput datasets, would also facilitate the integration of advanced AI systems, such as large language models (LLMs), into CHO cell engineering for biopharmaceutical production. Such tools could enable not only rapid data extraction and summarization, but also the prediction of gene functions and engineering outcomes to identify effective engineering strategies, tailored, for example, to protein-specific bottlenecks in biopharmaceutical production. As a result, even data from unsuccessful engineering attempts may become valuable for refining future strategies. Examples for the use of AI systems include: 1) A recent large-scale transcriptomic analysis of 890 monoclonal CHO cell lines, combined with machine learning, which led to the discovery of a novel stress biomarker;[206] 2) The development of LLM tools, such as CRISPR-GTP, for automated gene-editing experiment design, which supports selection of CRISPR systems, guides RNA design, delivery methods, protocol generation, and validation strategies[207] and could thereby facilitate CHO cell engineering; and 3) LLMs for knowledge synthesis, including automated data extraction from literature and AI-enhanced biomanufacturing workflows, enabling prediction of protein function, strain engineering strategies, and microbial cell factory optimization.[208]

Another limiting factor in advancing CHO cell engineering is that commercial entities often do not disclose data on the cell lines used or the titers achieved, instead keeping this information proprietary for competitive and strategic reasons.[209] Overcoming this limitation will require the establishment of standardized frameworks for data transparency and integrity, supported by policies and incentives that balance intellectual property protection with the need for open scientific exchange. Such measures would enable broader data sharing, fostering comparability, reproducibility, and more rapid innovation across the field.

As the development of mAbs progresses toward more complex derivatives such as Fc-fusion proteins, bi- and multi-specific antibodies, and single-chain diabodies, the demand to produce these often DTE molecules in sufficient quantities continues to rise.[2] Notably, within this dataset, CHO cell engineering for the production of antibody derivatives achieved a median improvement of 2.7-fold, exceeding the overall and mAb-specific median improvements (2.1- and 2.2-fold). This finding demonstrates that enhancing the production of DTE proteins is feasible. One possible explanation for the higher median improvement is that their initial production titers or qps are typically lower, leaving greater potential for improvement. Even though these general productivity-

enhancing strategies are successful in improving DTE protein production, additional product-specific challenges such as impaired secretion due to partially folded light chains can affect expression.[210] Thus, identifying the individual rate limiting steps, for example, during processing using technologies like fluorescence or electron microscopy, may be critical for enabling high-level DTE protein expression levels in the future.

An analysis of whether the engineering targets associated with the highest improvement folds in this dataset were subsequently repeated or adopted by other researchers leads to the following three conclusions: 1) C12orf35 KO remains a promising engineering target and a hotspot locus for site-specific integration, as supported by multiple follow-up studies;[170,211–215] 2) The concept of artificially introducing aneuploidy has, to the best of our knowledge, not been further pursued or published by other research groups; and 3) Enhancing sialylation via St6gal1 OEX has become a recurring research topic that has been cited over 13 times in the past five years (see for example references[216–218]). With two of the three approaches having a major impact on subsequent developments in the field, this analysis underscores that transparency and open scientific exchange are critical drivers of innovation in CHO cell engineering for biopharmaceutical production.

This comprehensive cross-study analysis also revealed that engineering low-producing CHO cell lines (defined as initial titer < 1 g/L and/or qp < 10 pg/cell/day) yielded a higher median improvement than engineering high producers (2.5- vs. 1.8-fold), although there was insufficient evidence to detect a statistically significant difference. Given the higher ER stress, metabolic burden, protein folding demands, and genomic instability associated with high-producing CHO cells,[219,220] validating engineering targets in high-producing lines remains essential. Academic-industrial collaborations will be crucial for evaluating the translatability of findings from low-producer models, like approximately half of the reports within this dataset, to industrial settings. Additionally, comparative omics approaches may help to elucidate the key differences between high- and low-producing cells, thus improving translatability prediction and enabling the identification of novel engineering targets.[221–223]

Finally, it could be shown that the maximal improvement fold reported per publication did not increase from 2013 to 2024. While this may seem surprising, given the rapid technological developments in CHO cell engineering, including CRISPR-Cas and high-resolution omics methods allowing, for example, the identification of new targets, the findings of this analysis are consistent with a prior analysis by Kuo et al. based on the data from Golabgir et al.,[187] which showed titer improvements plateauing around 2007.[7] This trend may be attributable to several factors: First, major drivers of productivity improvement since 1986, such as advances in bioprocess control strategies and cell culture media, were not covered within the scope of this review. Second, although publications on gene KO strategies in CHO cell engineering have steadily increased since 2011 (Figure 3(B,C)), single-gene OEX remained the most commonly reported engineering method in 2024 (47% of studies), despite its inherent limitations such as increased metabolic burden. An additional contributing factor may be that the majority of studies in this dataset reported on single target engineering (73%, see Supplementary Figure S3A), which, as discussed above, often yields only limited productivity improvements. Although less frequently reported, combinatorial engineering may represent the most promising path forward, particularly for addressing the expression challenges posed by increasingly complex biologics such as Fc-fusion proteins and multi-specific antibodies.

Looking ahead, the integration of combinatorial CHO cell engineering, high-throughput screening, omics-guided target discovery, and AI-driven tools such as LLM offer a promising path toward more predictive, efficient, and tailored strategies for producing increasingly complex biopharmaceuticals in CHO cells. To fully realize this potential, the adoption of standardized reporting formats and experimental model systems will be essential for enabling cross-study comparisons, data reuse, and improved translatability.

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Authors' contributions

A.S. conceptualized this review, conducted the systematic literature search and screening, extracted key performance data, conducted the analyses and wrote the original draft of the manuscript. A.T., K.O., A.P., and T.M. contributed to study conceptualization, reviewed and edited the draft manuscript. E.S., A.T. and K.O. supervised the work. All authors read and approved the final version.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT-4o in order to improve language clarity and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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