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Manipulating gene expression levels in mammalian cell factories: An outline of synthetic molecular toolboxes to achieve multiplexed control

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ABSTRACT

Mammalian cells have developed dedicated molecular mechanisms to tightly control expression levels of their genes where the specific transcriptomic signature across all genes eventually determines the cell's phenotype. Modulating cellular phenotypes is of major interest to study their role in disease or to reprogram cells for the manufacturing of recombinant products, such as biopharmaceuticals. Cells of mammalian origin, for example Chinese hamster ovary (CHO) and Human embryonic kidney 293 (HEK293) cells, are most commonly employed to produce therapeutic proteins. Early genetic engineering approaches to alter their phenotype have often been attempted by "uncontrolled" overexpression or knock-down/-out of specific genetic factors. Many studies in the past years, however, highlight that rationally regulating and fine-tuning the strength of overexpression or knock-down to an optimum level, can adjust phenotypic traits with much more precision than such "uncontrolled" approaches. To this end, synthetic biology tools have been generated that enable (fine-)tunable and/or inducible control of gene expression. In this review, we discuss various molecular tools used in mammalian cell lines and group them by their mode of action: transcriptional, post-transcriptional, translational and post-translational regulation. We discuss the advantages and disadvantages of using these tools for each cell regulatory layer and with respect to cell line engineering approaches. This review highlights the plethora of synthetic toolboxes that could be employed, alone or in combination, to optimize cellular systems and eventually gain enhanced control over the cellular phenotype to equip mammalian cell factories with the tools required for efficient production of emerging, more difficult-to-express biologics formats.

Introduction

Engineering cellular expression systems by genetic interference allowed the generation of cells with desired phenotypes as intended by the researchers. Eventually, this enabled the use of various cell types, (i) as model organisms to study and characterize fundamental biological mechanisms as well as mechanisms in diseases [1], (ii) as expression factories for recombinant products, such as biopharmaceutical molecules [2–4] or even (iii) as the therapeutic product itself [5,6]. Employing cellular systems for the recombinant production of therapeutic molecules started in the 1970 s [7] and had its commercial breakthrough with the availability of recombinantly produced insulin

Abbreviations: Fut8, a-1,6-fucosyltransferase 8; AAV, Adenovirus-associated virus; ADC, antibody drug conjugate; AID, auxin-inducible degron; BAX, Bcl-2-associated X; CHEF-1, Chinese Hamster Elongation Factor-1α; CHO, Chinese hamster ovary; CRISPRa, CRISPR activation; CRISPRi, CRISPR inactivation; dCas9, dead Cas9; degron, protein degradation system; dTAG, degradation Tag; EF, elongation factor; GOI, gene-of-interest; HEK293, Human embryonic kidney 293; KRAB, Krüppel-associated box; lncRNA, Long non-coding RNAs; m6A, N6-methyladenosine; MEAS, mutually exclusive alternative splicing; miRNA, micro RNA; MTX, methotrexate; ncAA, non-canonical amino acid; POI, protein-of-interest; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; SINE, Short Interspersed Nuclear Elements (SINEs); siRNA, small interfering RNA; SP, Signal peptide; synTF, synthetic transcription factors; TF, transcription factor; TIS, translation initiation sequence; uAA, unnatural amino acid.

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(Humulin®) from *Escherichia coli* by Genentech Inc. in 1982. As of today, recombinantly produced proteins are among the highest selling drugs on the market. Monoclonal antibodies are currently the dominant type of therapeutic protein and mammalian cell systems have emerged as the expression host of choice to produce them. In this class, Chinese hamster ovary (CHO) cells have become the most popular system, producing the majority of the approved products in recent years [8]. Although mammalian cells have been equipped with the basic machinery to produce high quality products, e.g. proteins with human-like post-translational modifications (PTMs), many novel, more difficult-to-express products require additional engineering of the cellular machinery to improve quantity and quality of the required biopharmaceutical [9,10].

Oversimplified, manipulation attempts of cellular systems can be divided into alterations of the cell's environment, e.g. optimizations of the culture medium or the bioprocess, or direct manipulation of the cells themselves, e.g. genetic engineering to alter the expression of certain genes. While the first approach has allowed substantial improvements of CHO cell productivities for several decades [11], the latter only gained ground in the last 10–15 years [2]. This is mostly owing to the lack of genomic information for CHO cells that only became publicly available from 2011 onward [12–16] as well as the lack of efficient molecular tools for genetic engineering. For instance, overexpression of beneficial factors was traditionally, and usually still is, accomplished by cloning the engineering gene under the control of a strong, in many cases viral, promoter, such as the human Cytomegalovirus (CMV) or the endogenous Chinese Hamster Elongation Factor-1α (CHEF-1) promoter [17]. Contrary to overexpression, gene knock-outs were initially difficult to accomplish, due to a lack of efficient, precise and feasible tools. Noteworthy, targeted gene disruptions, e.g. of the a-1,6-fucosyltransferase 8 (Fut8) gene using zinc finger nucleases, were also described [18,19], but could not find general application due to their complexity. Only with the discovery of RNA interference [20] and eventually CRISPR/Cas9 systems [21,22], did gene knock-downs and -outs become effectively available to the engineering community. In various cases, unregulated overexpression or knock-downs and knock-outs led to substantial improvements of recombinant protein productivity [2,23,24]. In many other situations (of which only a few make it into a publicly available report), no or only minor effects on the cellular phenotype could be detected [25]. The easiest, and probably in many cases correct, explanation for this is that a single gene alone simply does not impact the phenotype. On the other hand, inadequate or physiologically irrelevant expression levels of the transgene could result in nonmeasurable or even detrimental effects. Consequently, the actual beneficial effect(s) of the overexpression or removal might be masked by an overburdening of the cells coping with a stressful situation.

For antibodies the technology of random transfection and selection of a suitable production clone has become a platform technology of great success, with titers of 5–10 g/L achieved by now as a standard productivity level [26]. Thus, if it is possible to identify cells with such optimized protein expression machinery, one might question the necessity of optimization and fine tuning of expression levels of these genes to further enhance cell specific productivity. However, while cells are capable of achieving such fine tuning themselves, it is still a major effort to find and isolate such subclones. In typical screening efforts, thousands of subclones are analyzed to select the rare ones that perform adequately [27]. Being able to set and control gene expression patterns at just the right level for high productivity could potentially increase the chances of success and reduce the workload required to find suitable producers. In addition, over the last few years many more difficult to produce formats have emerged, such as bi- or even trispecific antibodies or therapeutic enzymes that frequently require either fine-tuned balancing of various heavy and light chains and/or additional cofactors that the host cell might not express at the needed level. Here again, tools and methods to fine-tune and optimize the expression level of specific parts of the cellular machinery to precisely match the requirements of the product could increase the manufacturability of such new therapeutic entities [10,28].

The increasing feasibility of next generation sequencing and mass spectrometry methods allowed the holistic characterization of mammalian cell factories and consequently led to the discovery of novel engineering targets as well as the improvement of the available resources [13,29,30]. What became apparent, not only from these investigations of CHO cells, but also from studies of other common expression systems, such as yeast and bacteria, is that optimal manipulation of cellular phenotypes often requires the modification of multiple genes as well as the fine-tuning of the respective gene expression levels, rather than a complete knock-out or maximum overexpression of a single gene [31,32].

To achieve their aims, synthetic biologists repurpose and optimize naturally occurring, molecular mechanisms from various organisms and implement them for their needs in the target expression system. Synthetic biology tools are already widely and regularly used to optimize and control phenotypes of microbial systems in the recombinant manufacture of desired products, such as bacteria (reviewed in [31, 33–35]), fungi (reviewed in [36,37]), or yeast (reviewed in [38–40]). Here, we discuss molecular and synthetic biology tools or strategies that were described to dynamically control gene expression levels in mammalian cell factories used for manufacturing therapeutic proteins. As CHO cells are the predominantly used cell line for this task, this review focuses mostly on studies that engineer this cell type.

Controlling gene expression levels

Subtle changes of expression levels of specific genes can have a profound impact on cellular phenotype, e.g. in disease or embryonic development [41–43]. Mammalian cells can therefore adjust and control their phenotype on several levels by (i) the transcription of genes, (ii) available messenger (m)RNA levels (post-transcriptional), (iii) translational efficiency and ultimately (iv) protein activity, stability and location (post-translational). In addition to these regulatory layers, the cells do not employ a single, but multiple regulation mechanisms per layer to precisely control the abundance and activity of their genes (Fig. 1). The many mechanisms and check points are important to protect cells from unintended changes in phenotype and eventually for the maintenance of their integrity and function in the (mammalian) organism.

For researchers that want to manipulate mammalian cells by genetic engineering to study and control phenotypic traits, these regulation layers come as boon and bane. On the one hand, they offer multiple entry points to develop tools for controlling gene expression strength. On the other, by adjusting expression levels of a gene solely at one of these regulatory layers, other layers might interfere and alter the expected outcome, e.g. high mRNA levels might not translate to high protein levels [44]. Therefore, in future engineering tasks, combinatorial use of toolboxes that act on different layers might be required to achieve this goal of phenotypic control.

Transcriptional regulation

The first possibility to interfere with the expression strength of a gene is by regulating the number of RNA molecules generated from the template DNA sequence, in other words regulation of transcription (Fig. 2). Transcriptional regulation tools in microbial systems have recently been reviewed [33]. While many mechanisms are similar to microbial systems, mammalian cells have also developed several additional, distinct mechanisms to control the transcription states and rates of their genes.

Promoters

The rate of transcription is mainly determined by the promoter located upstream of the gene's coding sequence. Varying the promoter

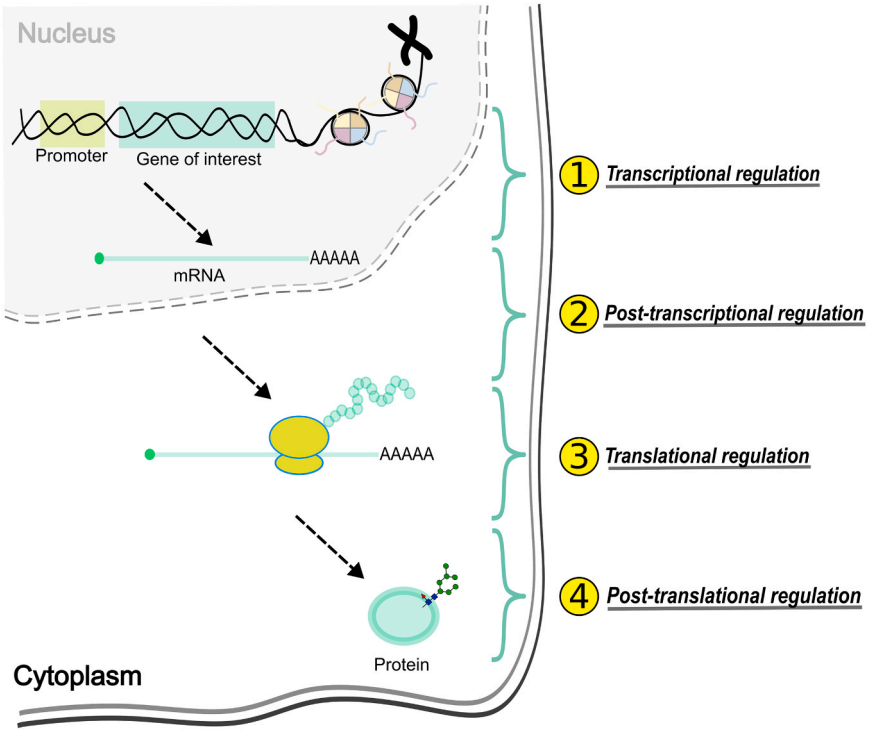


Fig. 1. Overview of (possible) regulation points for gene expression in a mammalian cell.

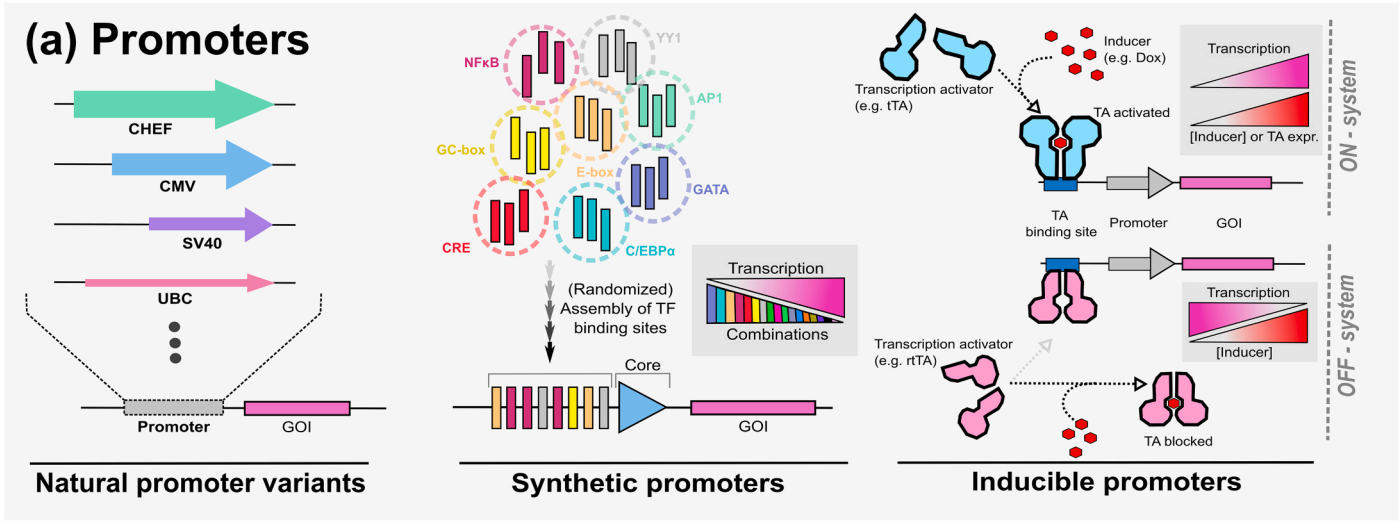
of the transgene to be expressed is the most widely used approach to control the expressional strength (Fig. 2a). Classically, natural promoters, i.e. those that were identified from biologic resources such as cellular or viral genomes, with variable strength have been used. Probably the most commonly used promoter in mammalian expression studies is the human CMV promoter, isolated from Cytomegalovirus, as it allows very high transgene expression [45]. To vary expression levels, other promoters such as the simian virus 40 (SV40), ubiquitin c, phosphoglycerate kinase 1, elongation factor 1 α or CHEF-1 are used [17]. Nevertheless, the search for new endogenous promoters or regulatory elements is ongoing, as endogenous sequences are believed to be less prone to gene silencing than e.g. viral sequences [46–50]. Thus, a long list of natural promoters is available for researchers to tune transgene expression levels. A pitfall of endogenous promoters is that they vary in size and are in many cases relatively large compared to viral promoters. This often hinders their direct use in recombinant protein expression, which, however, can be overcome by promoter engineering [50,51]. Two recent reviews discuss the engineering and use of natural as well as synthetic promoters for CHO cell engineering [17,51].

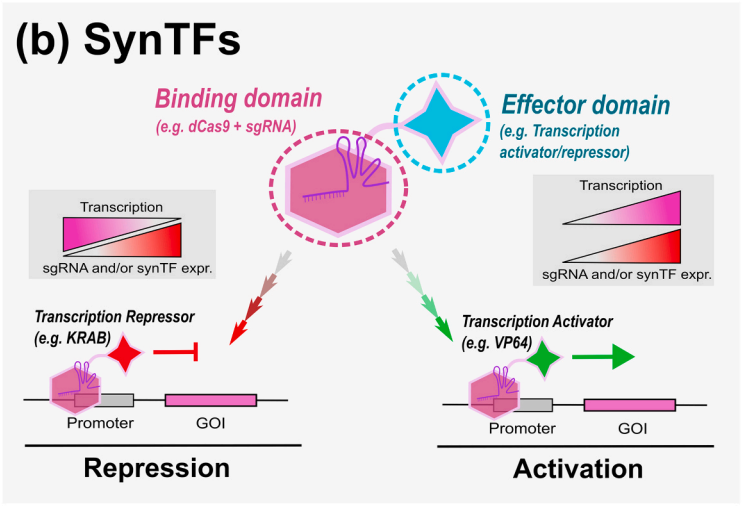
Besides natural and endogenous promoters, synthetic promoters have become increasingly important to control expression rates of transgenes. The design and construction of synthetic promoters can either be achieved by mutating the DNA sequence of regulatory elements in the promoter, such as TATA or CAAT boxes, or by combining multiple parts to form a new promoter, e.g. transcription factor (TF) binding sites in the proximal promoter with a core promoter [17,51,52]. Uses of synthetic promoters in CHO cells were already described in the 2000 s [53,54]. With increasing feasibility of DNA synthesis and also next generation sequencing, library designs of synthetic promoters became possible and allowed the screening of several hundred promoter variants [55–57]. Eventually, this enabled dynamic control of expression levels from comparably small promoters.

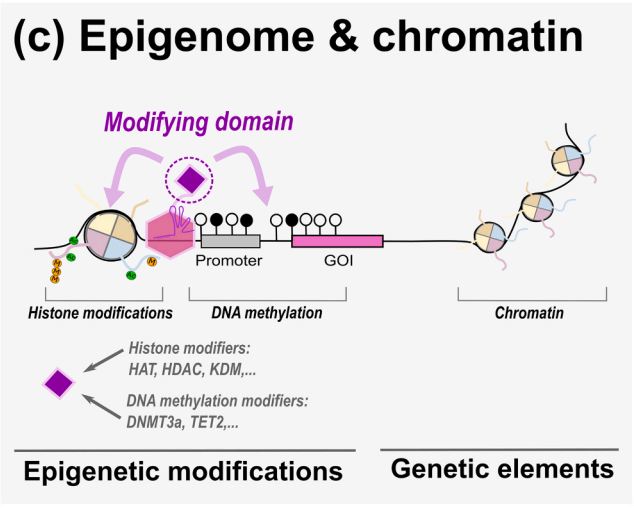
All of the above-described promoters are so-called constitutive promoters. While in many cases regulated, but constant expression is desired, in many other cell line engineering scenarios inducible expression is required and favored, e.g. when recombinant protein expression should be aligned with growth phases in a production process [58], or when the expression level of an engineered gene should respond to cellular state and environmental conditions[59]. Many inducible systems have been described and established for CHO and other mammalian systems (Table 1). Classically, inducible systems rely on the presence (or absence) of a small chemical compound that facilitates binding or dissociation of a specific TF to or from the promoter and subsequently activates or blocks transcription of the gene of interest (GOI). Probably the most widely used mammalian inducible system is based on tetracycline-mediated induction, but other systems such as the cumate or streptogramin and many more were also described to function in CHO cells (Table 1) [17,60]. However, many of these are only ON/OFF types and do not allow fine-tuned control of transcription. To add another level of control, the activating or repressing molecule is often used to control the expression of a chimeric TF-fusion that specifically binds to the promoter of the actual GOI and controls its expression. Combining multiple inducible systems eventually allows the construction of more complex expression systems in mammalian cells, so-called “genetic circuits”. By this it becomes possible to install certain phenotypic traits in a cellular organism and specifically control it by extrinsic stimuli [61]. The use of sensor elements is a novel way to equip CHO cells for production under suboptimal bioprocess conditions. Cloning of hypoxia-responsive elements, for example, allowed the induction of gene expression under hypoxic conditions [62]. Other sensor elements that can drive gene expression might be used in the future to autonomously adjust gene expression in response to culture conditions and thus could allow fine-tuning of cell phenotype in a self-regulatory fashion.

Synthetic transcription factors

To execute their function, native but also synthetic promoters rely on binding of endogenous TFs. The expression rates mediated by these promoters are heavily impacted by the number and combination(s) of specific TF binding sites in the promoter sequence as well as the internal expression levels of the TFs themselves. In order to harness this level of transcriptional control, so-called “synthetic transcription factors







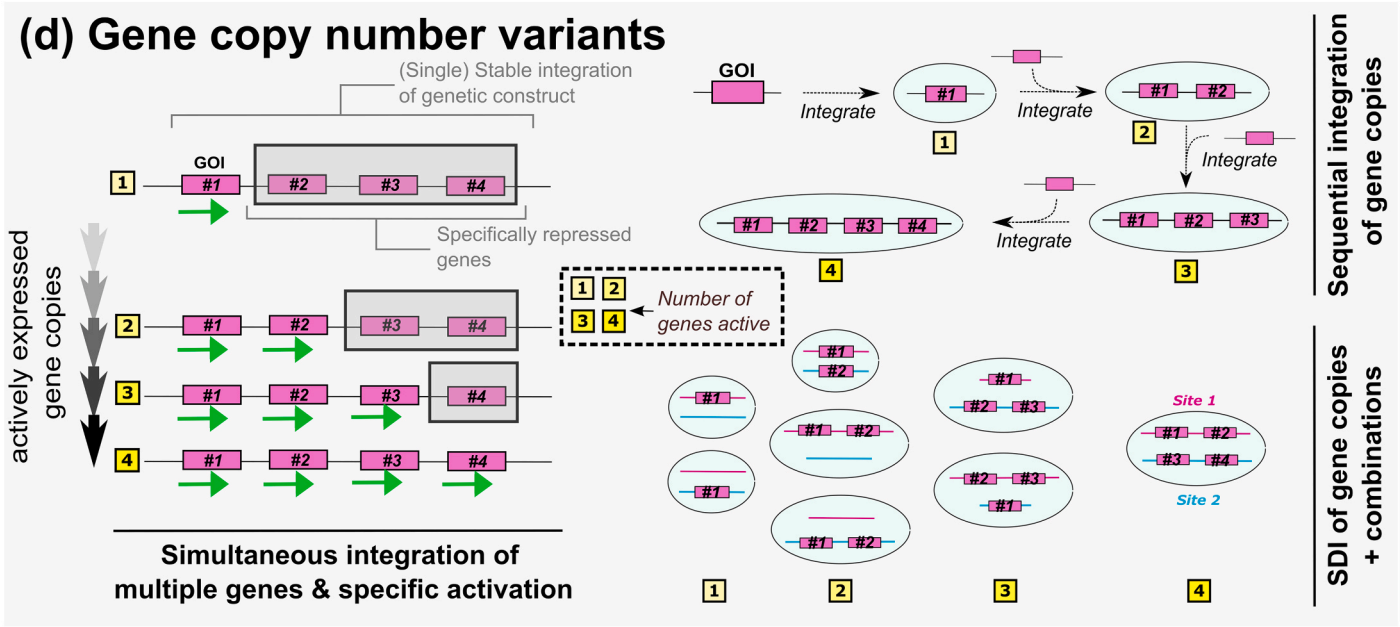


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Fig. 2. Transcriptional regulation tools. (a) Promoters as tools to tune transcription strength. Left: Examples of natural promoters widely used in recombinant protein production in CHO cells. Size of the arrows indicate promoter strength. GOI = gene-of-interest. Middle: Assembly strategy of synthetic promoters. Bars in different colors represent distinct transcription factor (TF) binding sequence. Right: Principles of ON- and OFF-systems of inducible promoters. Dox = Doxycycline. TA = transcriptional activator. (b) Principles of synthetic transcription factors (synTFs). (c) Epigenome and chromatin modification strategies to alter gene expression levels. Black and white circles indicate methylated an unmethylated cytosines. (d) Principles to rationally control gene copy numbers. For easier interpretation GOI copies are numbered by #1–4. Left: Integration of multiple genes and sequential activation. Method reported in [119]. Top right: Sequential integration of GOI copies as described in [118]. Bottom right: Site-directed integration (SDI) of GOI copies in multiple integration loci. Here, two GOIs are integrated per integration site but more are possible.

(synTFs)" have been developed (Fig. 2b). These synTFs consist of a binding domain, that can specifically recognize and bind to a defined target DNA sequence, and an effector domain, that can either promote or repress transcription. Thus, control of – in principle – any GOI expression level by synTFs can be executed by targeting the binding domain to any specific genomic location and selecting an appropriate effector domain. In particular, the introduction of a catalytically inactive Cas9 protein (or dead Cas (dCas9)) and its fusion to different effector domains, has allowed, among other modifications, the activation (CRISPRa) [63–65] or inactivation (CRISPRi) [66] of precise loci thanks to the design of customized sgRNAs. Although several tools that direct TFs to a desired genomic locus, such as TALEs or zinc finger proteins, were developed, dCas9-based tools are the most commonly employed technology for CRISPRa/i, also in CHO cells, due to their flexibility and ease of use [67–73]. Compared to conventional all-or-nothing approaches such as knockouts and transgenic overexpression, CRISPRa/i can be potentially used to modulate gene expression in a broader way and better recapitulate physiologically relevant changes in gene expression levels.

Compared to classic plasmid overexpression, CRISPRa can selectively activate a GOI from its original chromosomal locus [74], which guarantees that the gene is correctly transcribed and localized in the region where it performs its function. This is extremely important in the case of non-coding transcripts where the transcription of the proper native sequence and its localization in the correct compartment is crucial to ensure their function [75,76]. Different CRISPR architectures such as the synergistic activator mediator (SAM) system [77,78] and the Sun-Tag system [79] can be used to increase CRISPRa/i efficiency. Various CRISPRa systems have been developed to promote transcription. Detailed information can be found in [80,81]. Most prominently, CRISPR activators such as VP64 [64,82,83], VP64-Rta [84], VP64-p65-Rta (referred to as VPR) [84], VP192 [85], etc [86] with a various number of repeat units and orientations have been used. The functionality of CRISPRa was demonstrated in CHO cells, for which the expression of two naturally silenced genes, *Mgat3* and *St6gal1*, were successfully activated. Consequently, a more human-like glycan structure of recombinantly produced proteins could be achieved [67].

On the other hand, knock down of a gene by CRISPRi is induced by binding of the CRISPR complex to the DNA sequence complementary to the sgRNA(s) resulting in a steric block that halts transcript initiation and/or elongation by RNA polymerase and ultimately leads to the repression of the target gene. To further improve the strength of the downregulation, dCas9 repressors have been developed by C-terminal fusion of the Krüppel-associated box (KRAB) or a bipartite repressor domain, KRAB-MeCP2 [87], to dCas9. CRISPRi has been successfully used in CHO cells to temporarily downregulate the endogenous expression of apoptotic genes *Bak*, *Bax*, and *Casp3* or other genes involved in glycosylation (*St6gal1, Fut8*) and transgenes under the control of the CMV promoter [69,73]. On the other hand, to increase recombinant protein production, CRISPRi was applied to suppress recombinant *Dhfr* gene transcription during MTX selection to impose additional selective pressure [72]. However, as for CRISPRa, the transcriptional effects are only transient and are not maintained for longer than a few days if the synTFs effectors are not stably overexpressed themselves. Thus, these tools are, in their current form, mainly usable to investigate gene-function in mammalian cells.

In a broader definition, CRISPR-based targeted DNA methylation and demethylation also execute CRISPRi/a functions by turning transcription off or on [69,70]. However, in contrast to the dCas9-tools discussed above, they stably change expression of targeted genes, since the changed DNA methylation signature is stably copied during replication. In fact, most dCas9 tools are direct epigenetic modifiers and not only function as transcriptional entities but also affect the epigenetic environment. Due to their interaction with endogenous epigenetic factors, a dissection of direct or indirect effects on the transcriptional machinery is not always possible. On the other hand, general modulation of the epigenome and harnessing its impact on gene expression is a promising technology that not only enables a better understanding of CHO cell biology, but can be leveraged for cell line engineering purposes.

Epigenome modulation

Epigenetic modifications, such as DNA methylation or various post-translational modifications of histone tails, tightly control gene expression of endogenous genes or transgenes in mammalian cells by modulating chromatin accessibility and affecting the transcriptional machinery. The alteration of epigenetic marks allows mammalian cells to adjust gene expression levels upon environmental changes or during different cell cycle phases [88–92] (Fig. 2c). While DNA methylation fixes gene expression either in an ON/OFF state and is stably inherited to daughter cells, histone modifications are more dynamic and have distinct effects on transcription [69,70,90,93]. Importantly, the abundance of histone marks or different combinations can affect the level of gene expression rather than resulting in a unidirectional expression/non-expression state. Epigenetic mechanisms in CHO cells and harnessing thereof for cell line engineering efforts have recently been reviewed [94]. In principle, epigenetic marks can be formed or removed by epigenetic writers and translated into altered transcription by the corresponding readers. Of advantage is the fact that introduced modifications can be removed again (or vice versa), thus restoring the original expression pattern of the respective gene [69,70] without altering the underlying genomic sequence, which mitigates potential undesirable effects of classical gene knock-in or knock-out. Global, but random, epigenetic changes that result in multiple, but undirected alterations in the gene expression pattern have been investigated. Here, modulation via addition of small molecules, e.g. short-chain fatty acids such as sodium butyrate, has been commonly used to boost protein production in CHO cells [95–105]. Cytosine analogues, such as 5-azacytidine, have also been used to investigate the effect of DNA methylation on gene expression [106]. The randomness of these approaches – various genes will be impacted by these techniques – results in a wide range of phenotypes with heterogenous transcriptomes and epigenomes. While at first glance a seemingly undesired effect with respect to phenotypic stability, the diversification offers the possibility to isolate cell clones that have acquired improved gene expression patterns due to the epigenetic modulation which is of interest, in particular during the screening phase of cell line development. Intriguingly, knock-down of key epigenetic enzymes (i.e. DNA methyltransferases (DNMTs) and TETs) by repeated siRNA transfections, resulted in increased phenotypic variation in a cell pool and allowed selection of clones with improved transgene expression [107]. While this strategy can be applied to isolate better production clones, it remains an uncontrollable technique, especially because the exact mechanisms and the network of epigenetic

Table 1
Summary of molecular toolboxes classified according to their main regulation mechanism.

Principle	Toolbox technology	Cell lines tested	Reference
Transcriptional regulation			
	Gene copy variants	CHO	[114,116]
		other	[115]
	Promoters		
	Synthetic promoters	CHO	[51,262–264][50]
		HEK293	[49,51,52,265–272]
		other	[49,51,52,265,266,269,272]
	Inducible promoters	CHO	[45,46,57,163,273–292]
		HEK293	[163,270,271,287,288,292–305]
		other	[163,274,275,287,288,292,294,295,298,299,302,306–313]
	SynTFs		
	dCas9-fusions	CHO	[64,65,67,66,68–70]
		HEK293	amongst others [63,314,315],[61,65,79–81,83,261,316–320]
		other	amongst others[74,81,321,322]
	ZF-fusions	CHO	N/A
		HEK293	[271]
		other	[323–327]
	TALEs-fusion	CHO	N/A
		HEK293	[328], [272,329–333]
		other	[272] [332,334,335]
	Other factors	CHO	[161,162,257,276,336]
		HEK293	[161,162,257,268,269,290,299,303,336–339]
		other	[161,162,257,269,290,299,307,337–339]
Post-transcriptional regulation			
	Alternative splicing	CHO	[169]
		HEK293	[168,169]
		other	[168,169]
	RNAi	CHO	[161–163]
		HEK293	[20,161–165,340]
		other	[161,163,165,340,341]
	Riboswitches	CHO	[181]
		HEK293	[164,172,174–176,179,181,342–348]
		other	[174,176–178,181,345–349]
	CRISPR/Cas systems	CHO	[185,214]
		HEK293	[183,184,214,350,351]
		other	[184,214]
	Other	HEK293	[129]
		other	[129]
Translational regulation			
	TIS	CHO	[10,212,218,352]
		HEK293	[212,353]
		other	[212,353]
	Signal peptides	CHO	[226,232–238][228–230,239]
	uORF	CHO	[212,214]
		HEK293	[212–214]
		other	[212–214]
	RNA structures	CHO	[199]
		other	[196,198]
	RNA structures + ligand	HEK293	[165,354–356]
		other	[165,357–360]
	IRES	CHO	[208,209,352,361–363]
		HEK293	[208,209]
		other	[208]
	uAA	CHO	[243,245,364–366]
		HEK293	[365,367–372]
		other	[365,373]
	Other	CHO	[374]
		HEK293	[375]
		other	[375]
Post-translational regulation			
		Targeted degradation	
	Destabilizing domains	CHO	[251,257,376]
		HEK293	[251,376–378]
		other	[251,256,257,376–378]
	Self-cleaving peptides	CHO	[379]
	Protein splicing	CHO	N/A
		HEK293	[254]
		other	[254,255]

regulation in CHO cells and its impact on protein production stability have not been fully elucidated yet. Initial studies using dCas9 technology to guide epigenetic effectors to endogenous promoters in CHO cells, next to modulating expression of specific genes, were able to confirm the prominent role of DNA methylation in fixing hereditary gene expression patterns in CHO cells [70]. Moreover, the autologous remodeling of histone modifications upon changes of the DNA methylation status of endogenous and exogenous promoters was observed [69]. Thus, CRISPR-based targeted epigenetic editing offers the possibility to understand the contribution of epigenetic marks on gene expression and

the advantage to selectively change expression of individual genes. Consequently, random and targeted epigenetic editing represent complementary methods to modulate gene expression in CHO cells and first and foremost to understand the epigenetic impact on the cell's phenotype. Nevertheless, further investigations are necessary to dissect the complex network of epigenetic interactions in order to understand the impact of different cultivation techniques, media additives and general stressors (e.g. temperature and pH shifts, freeze/thaw, media adaptation) on gene expression and subsequently on CHO cell heterogeneity. One case that has been demonstrated is the fact that subcloning itself, as a stressful situation to cells, induces changes in cell DNA-methylation pattern, which explains the well-known fact that subclones generated by re-subcloning of a subclone will develop a wide range of phenotypes that do not necessarily match that of the parental subclone [108].

Eventually, tools to fine-tune protein expression via epigenetic modifications to specific levels rather than turning them on or off would be desirable for gaining precise phenotype control in mammalian cell lines. So far, these have not been described, as far as we are aware.

Gene copy variants

Changing the number of gene copies available to the cells for transcription is an important aspect affecting expressional levels (Fig. 2d). From a biological point of view, this is not a mechanism that really allows dynamic and rational control of the expression strength by the cells, but is rather a consequence of faulty genome replication and integrity that drives evolutionary development and diversification in a brute-force way. Whether gene copy numbers really correlate proportionally with the expression level of the respective gene depends on the gene itself and/or the genomic environment [109]. Still, several gene amplification systems, most importantly the dihydrofolate reductase (DHFR) / methotrexate (MTX) or glutamine synthetase / L-methionine sulfoximine systems, were developed and found broad application in mammalian cell line development campaigns [23,110,111]. While these classic gene amplification systems enabled efficient generation of CHO cell producer lines, they do not allow precise control over gene expression levels as the precise number of active genes cannot be controlled rationally. Obtaining a suitable producer cell line therefore is likely dependent on a combination of coincidental, but advantageous alteration of gene-of-interest (GOI) copy numbers and the endogenous transcriptome [112]. To isolate such a clone, efficient high-throughput screening technologies are needed. The development of site directed integration technologies, such as CRISPR/Cas9 or recombinase mediated cassette exchange allowed the precise integration of the GOI(s) into a defined locus of the genome (reviewed in [113]). Combined with tailored analysis methods [114–116] it also became possible to rationally control the number of GOI copies that are integrated, which also allows to study gene dosage effects in CHO cells and eventually improve the recombinant productivity to industrially relevant levels with little clonal variation among the isolated cell lines [117]. For HEK293 cells, successive lentiviral integration steps of the GOI resulted in a consistent increase in the specific productivity with each integrated copy (up to 3 copies tested) [118]. To allow a step-wise activation of such transgene copies, stably integrated repressed transgenes can be activated by CRISPR/Cas9-mediated deletion of repressor elements that block gene expression in CHO cells. This approach allowed the specific activation of expression of single or multiple genes in any combination [119] and can for instance be employed to study various combinations of different engineering factors to find optimal expression ratios. Additionally, it can also be used to successively increase the number of actively expressed genes (of the same or similar genes) and thus study the response of cells to gene dosage.

LncRNAs

Long non-coding RNAs (lncRNAs) are RNA transcripts longer than 200 bp that do not encode for any protein. Although thousands of these molecules have been annotated, only a few of them have been characterized and associated to a variety of roles in the regulation of transcription [120], translation [121] and chromatin states [122–124]. For more detailed review of functional roles of lncRNAs, the reader is referred to the review of Statello et al. [125]. Given the variety of levels at which lncRNAs are active, they represent an interesting potential tool to modulate the cell transcriptome and translatome by operating at different subcellular compartments. A very promising mechanism that can be exploited to fine-tune gene expression, is the triplex structure that homopurine/homopyrimidine regions of some lncRNAs are reported to form with the promoter of their target gene [126–130]. Ideally, the region responsible for triplex formation could be exchanged to target any GOI. Others instead have used the triplex-forming region of the lncRNA *Malat1* to overcome mRNA destabilization and enable simultaneous expression of protein and CRISPR arrays on the same transcript [131]. Similar to triplex-forming lncRNAs, another category of lncRNAs have been reported that contain regions called Short Interspersed Nuclear Elements (SINEs). Thanks to their complementarity to specific mRNA sequences, they seem to upregulate the translation of specific targets (therefore called SINEUPs) [121,132]. Recent work also underlines the possibility that lncRNAs are localized in exosomes [133] or other specific cellular and nuclear compartments [75,76], contributing to the nuclear architecture, formation of higher order chromosomal structure and therefore control of gene expression [134,135]. It remains to be explored how this finely controlled 3D organization exactly works and whether by targeting a specific lncRNA one could promote the assembly of specific chromatin structures that in turn would affect the expression of the genes involved in the same domain [76,135].

Noteworthy, the concept of using long, anti-sense RNAs to regulate gene expression has already been employed in the 1990's. In CHO cells, anti-sense RNA was for instance employed to reduce sialidase expression in CHO cells and thereby increase the sialylation on a recombinant DNAse [136]. These anti-sense RNAs can modulate gene expression on multiple layers, from transcription to (m)RNA stability and translation [137].

In CHO cells, lncRNAs were investigated in several publications in recent years [91,138–141]. Intriguingly, clear correlations between differential expression of coding genes and lncRNA have been described, indicating an important connection between these two classes. Hence, controlling expression of certain lncRNAs could open up new possibilities to alter gene expression levels of endogenous genes and consequently a novel strategy to alter cellular phenotypes. In the study by Novak et al., many lncRNAs that were identified to correlate to process-relevant properties such as productivity were not able to alter the phenotype. However, the good correlation could make them valuable tools as biomarkers. For one specific lncRNA, a significant change in cell size and specific productivity was observed after gene knockout which would indicate that at least some lncRNAs have potential for cell engineering [142].

It is noteworthy that lncRNA research and its application are in their infancy and specific, curated information such as correct annotation and functionality in CHO cells (and most other mammalian systems) is still missing. Therefore, establishing the rational use of lncRNAs as a molecular tool to alter gene expression levels of their targets in CHO cells or other mammalian cell lines used in recombinant protein production in a predicable way has so far not been reported.

Considerations for cell line engineering

Together, several mechanisms to control the transcription rate of a GOI exist and are available for mammalian cell engineering. Importantly, transcription is dependent on many cell internal factors. For instance, the integration locus and its genomic environment (enhancer, insulators, chromatin state,…) strongly impact the expression rate of the integrated GOI. Consequently, different GOI expression strengths can be observed from the same promoter, but different integration loci [143, 144]. The development and application of efficient site-directed integration tools for stable transgene integration will help to reduce such

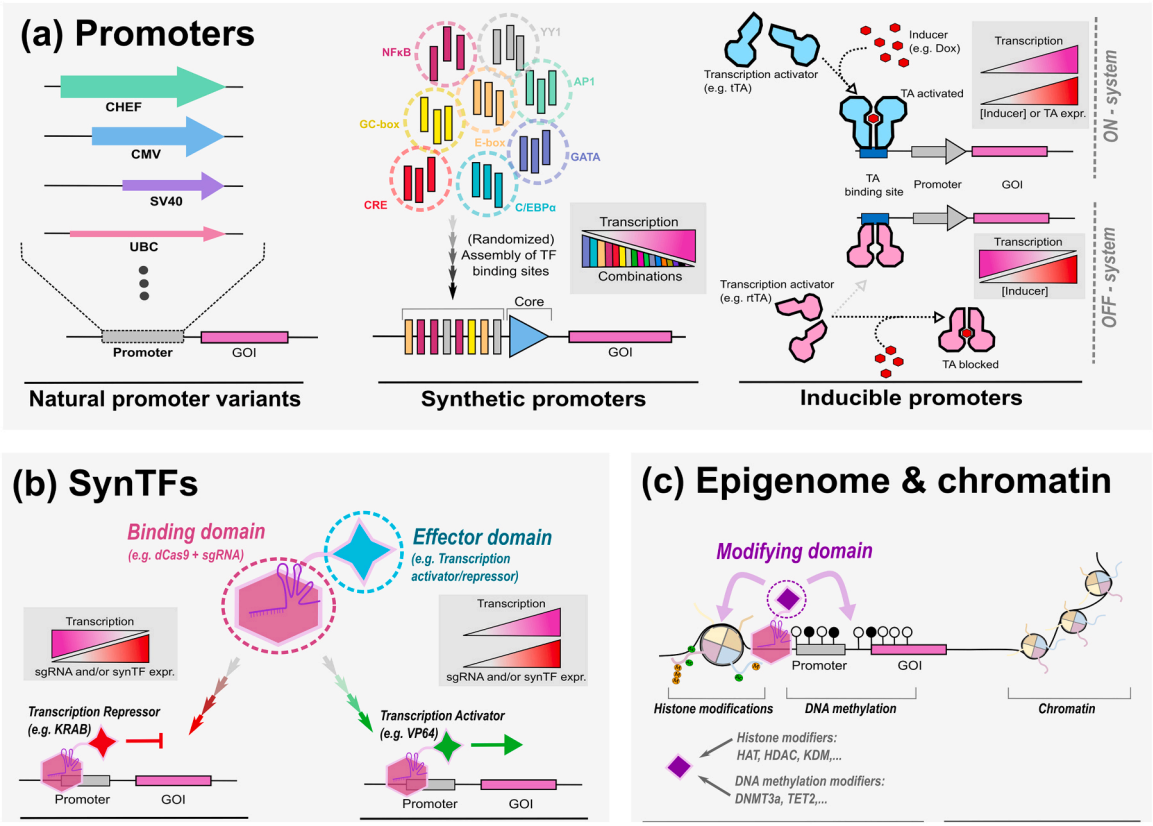

Fig. 3. Posttranscriptional regulation toolboxes. (a) mRNA processing alterations with riboswitches to generate alternative mRNA variants as described in [176]. (b) RNA(i) interference tools. Top: Heterologous introduction of shRNA (can also be stably integrated) or miRNA mimics into the cellular systems. Mature miRNAs form the RISC with regulatory proteins and knock-down their respective target(s). Middle: Reduction of endogenous miRNA with heterologously introduced miRNA sponges. Bottom: Varying miRNA binding sites in target GOI to control its expression level. (c) RNA-based gene switches to control mRNA stability. A ligand that specifically binds to the aptazyme can either induce a conformational change of the RNA-based gene switch and consequently mediate self-cleavage and hence destabilization of the mRNA (Top; OFF-system) or stabilize the mRNA (bottom, ON-system). (d) CRISPR/Cas13 system can impact mRNA stability by inducing target degradation or with the use of Cas13-fusion proteins (e.g. with specific RNA modifiers) can introduce modifications to the mRNA that alter the expression level and/or stability.

impact. Furthermore, as TF expression levels can vary between cell lines, the same promoters used in different cell lines potentially yield different transcriptional output indicating that development of a universally applicable transcription regulation tool for mammalian cell lines might be hard to achieve. Still, promoters offer very versatile toolboxes that range from natural to synthetic and even inducible systems. Likely, the biggest advantage of using promoters as a measure to regulate gene expression levels is that promoters are very well studied and described. For basically all prevalent and regularly used cell types researchers can choose from a variety of promoters with different potencies, inducibilities or even tissue specificities. On the other hand, usage of different promoters, currently restricted to the expression of transgenes, requires the integration of expression vectors into the host genome and does not allow for further adaptation of the expression level.

SynTFs, on the other hand, are actively modulating a highly conserved regulatory layer and thus are likely to function similarly in various mammalian expression systems. Intriguingly, synTFs can be employed to basically target any genomic loci. This enables researchers to not only modulate expression levels of artificially introduced transgenes, but to directly modify expression strengths of (any) endogenous gene(s). Additionally, synTFs can be multiplexed and therefore allow to modulate expression of multiple genes simultaneously. Still, most synTFs themselves require to be introduced into the cellular system to either transiently or stably alter expression of the target gene. Since, efficient usage of cellular resources, such as amino acids, ATP, etc., is desired for optimizing recombinant protein production in CHO or any other cellular expression system. Therefore, recombinant expression of the synTF on top of the recombinant product itself needs to be carefully considered.

LncRNAs have great potential to site-specifically induce transcriptional alterations, also of endogenous genes. Contrary to synTFs they do not require heterologous overexpression of proteinogenic factors. Hence, they do not consume important resources, for instance amino acids. Currently, the downside of lncRNAs, especially in CHO cells, is that information on lncRNAs and their functionality is severely limited since the prediction of triplex formation and their experimental validation is difficult in living cells.

From an engineering perspective gaining control of transcription would be most desired, as the cells solely use up resources that are required for the targeted transcript level(s) of the GOI at the optimum level. However, transcription is the first of many steps in the biosynthesis of proteins. Therefore, expression levels of the mature protein might be impacted by many other mechanisms along the road, meaning that transcription levels do not necessarily correlate with the expression level of the mature protein. Consequently, molecular tools that allow regulation of these other, downstream steps will also be required to ultimately control the expression level and the cellular phenotype.

Post-transcriptional regulation

mRNA processing

Once the transcriptional process is initiated, mammalian cells can actively control the mRNA abundance by various regulatory levels (Fig. 3a). Firstly, the transcribed mRNA strand is modified with N6-methyladenosine (m6A) being the most frequently studied base modification with impact on RNA metabolism [145]. Consequently, the depletion of m6A-readers associated with mRNA decay (i.e. 5-member YT521-B Homology domain-containing family proteins) resulted in increased recombinant protein expression in CHO cells [146]. For protein-coding genes, RNA modification usually also includes capping of the 5`-end with 7-methylguanosine, splicing and addition of a poly(A) tail at the 3`-end. For example, using different poly(A) elements resulted in increased recombinant protein expression[147]. While the modifications at each end of the mRNA are generally required for mRNA export out of the nucleus and to prevent degradation, splicing allows the cells to control the genetic content that is to be translated. Mammalian cells can produce multiple splice variants from one mRNA, a process known as "alternative splicing". Molecular tools specifically allowing to control alternative splicing in mammalian cells are scarce, but some exist (see 0). After splicing, the quality of the generated mRNA is assessed by cellular mechanisms such as nonsense-mediated decay, to identify and remove any faulty mRNAs [148]. To the best of our knowledge, there are no studies describing the intentional use of these mechanisms to rationally control transgene expression. As this is a global mechanism that affects all mRNAs in the cell, one would need to include specific sequence features, such as a pre-mature stop-codon, in defined sequence stretches that can be excluded upon an input signal. Also, mRNA export from the nucleus is tightly controlled in mammalian cells, but so far has not been used to control exogenous transgene expression.

RNA interference

One of the most common regulation systems of mRNA levels in mammalian cells involves guiding of regulatory proteins to their target mRNA via small, non-coding RNAs that bind to complementary RNA sequences, a process commonly summarized under the term of "RNA(i) interference" (Fig. 3b). The two most prominent of these RNAs are small interfering (si) RNAs and micro (mi) RNAs, that are typically ~ 19–22 bp long. Although they differ in their formation process, they both are eventually incorporated into the RNA-induced silencing complex (RISC), that consists of siRNA or miRNA and regulatory proteins, most importantly the Argonaute protein. siRNAs usually bind with their whole sequence anywhere in the mRNA sequence and solely induce cleavage of the mRNA by the RISC [149]. miRNAs on the other hand can mediate repression of translation, induce mRNA degradation or directly cleave the mRNA strand. With the description of the RNAi system it became possible for scientists to design short complementary siRNAs and study their effect on the cellular phenotype. The further development of short hairpin (sh)RNAs enabled stable and long-term knock-down of target genes [150]. siRNAs are usually employed to knock-down a single, known target. They were successfully employed in CHO cell engineering to improve productivities or product quality [151], i.e. by knock-down of pro-apoptotic genes [152–154], glycosyltransferases [155,156], metabolic [157] or epigenetic [107] factors. Moreover, siRNA knock-out screens were conducted to identify novel engineering targets [158,159]. miRNAs on the other hand often target multiple different mRNAs and thus can regulate various genes [160]. As miRNA target prediction for CHO cells is difficult, due to a lack of target prediction tools, many mRNA targets of specific miRNAs remain unknown or are only assumed, based on studies and tools available for other organisms [161]. Still, miRNAs have been popular engineering targets in CHO cells [2162–164] because they do not burden the cell's translational machinery and therefore remain interesting tools to regulate multiple genes [165]. Knock-down of endogenous miRNAs can be accomplished by short, anti-sense oligonucleotides, or more advantageously by using so-called "miRNA sponges". These sponges are long oligonucleotides that harbor multiple binding sites for the target miRNA and thereby reduce the miRNA's activity in the cell by binding them to the sponge. miRNAs sponges have been used in CHO cells to improve recombinant productivity, for instance to by depleting miR-7 activity [166,167]. A protocol describing how a miRNA sponge expression can be controlled using TET-ON system is also available [168].

siRNAs have been adapted to regulate gene expression in an extrinsically controlled manner. This is usually accomplished by combination of RNAi with other regulation systems, e.g. repressors [169], inducible promoters [170], genetic loops [171], riboswitches [172] or miRNA-responsive elements [173]. Multiple other tool set-ups were described in literature for dynamic control of gene expression in other mammalian systems (Table 1). Together, these reports demonstrate the applicability of RNAi tools for dynamic, tunable regulation of gene expression levels. Interestingly, in most cases RNAi tools are combined with other regulatory toolboxes to gain tight control over the regulated gene.

RNA-based gene switches

mRNA levels can also be controlled by specific RNA secondary structures in the untranslated regions, so-called "RNA-based gene switches" (Fig. 3c). Riboswitches are naturally occurring RNA structures that can respond to the presence of specific small molecules, such as metabolites or antibiotics but also large molecules (e.g. proteins) and subsequently execute a conformational change. These changes in the RNA structure consequently expose or hide regulatory sequences resulting in either transcriptional or translational effects. In prokaryotes, riboswitches are therefore often found in the 5`-UTR where they govern the accessibility of the ribosome binding site and consequently regulate translational rates of the downstream gene [174]. In mammalian systems, only protein-responsive riboswitches were described so far to occur in nature [175]. Still, synthetic riboswitches that respond to small molecules, as for example tetracycline, were developed to regulate splicing of pre-mature mRNAs by controlling accessibility of splice sites in mammalian cells [176]. On the other hand so-called "mutually exclusive alternative splicing" (MEAS) that relies on the incorporation of mutually exclusive exons in the RNA isoforms based on the intron sequence between the two exclusive exons could be used to modulate expression levels of two independent genes [177]. Although this MEAS technology was also shown to work in CHO cells, to the best of our knowledge no report exists that applies alternative splicing to engineer CHO cells for improved production characteristics.

Further efforts of the synthetic biology community enabled the generation of so-called "aptazymes", that combine self-cleaving RNA structures, known as "ribozymes", with metabolite/molecule sensing RNA structures, so-called "aptamers" [178]. Contrary to the natural riboswitches, aptazymes induce cleavage of the mRNA strand they are located on and could therefore theoretically be placed anywhere in the mRNA but are preferentially put into the 3`-UTR, where their presence does not interfere with translational initiation, as it might at the 5`-UTR. Riboswitches and aptazymes can be designed in two ways. OFF switches generally repress GOI expression upon presence of the signaling molecule and activate it in its absence. Contrary, ON switches work the other way around [178]. For more detailed information on aptazyme functionality, the reader is here referred to the excellent recent reviews of Ausländer and Fussenegger [179] as well as Yokobayashi [178].

Aptazymes-based applications, besides showing their functionality with reporter genes, were reported to control expression of viral genes [180–185] or T-cell proliferation [186] in mammalian systems [187]. Although CHO cells were among the first organisms in which riboswitch structures were described [188], so far they did not find broad applications in engineering attempts of these cell factories. Ausländer and colleagues tested and established protein-responsive RNA structures in multiple mammalian cells, in which CHO-K1 were also included [189].

A guanine-responsive aptazyme was employed by Strobel et al. to reduce toxic transgene expression, e.g. of the proapoptotic Bcl-2-associated X (BAX) protein, during Adenovirus-associated virus (AAV) production in HEK293 cells. This study highlights the potential of such elements in recombinant manufacturing of therapeutic products.

RNA targeting with CRISPR

The term CRISPR is oftentimes used synonymously for the CRISPR/Cas9 system originally identified in *Streptococcus pyogenes* which has been widely used, inter alia, to introduce double-stranded DNA breaks in a plethora of cell lines and organisms. Since the first hallmark publication in 2012 [21], several other CRISPR systems have been discovered with different molecular architecture and various functions. Among those, the CRISPR/Cas13 system (also known as C2c2) has been identified as an RNA-targeting ribonucleoprotein complex that cleaves RNA transcripts site-specifically dependent on the targeting guide RNAs [190, 191] (Fig. 3d). Of the initially described types, the RfxCas13d (or CasRx) subtype showed enhanced knock-down efficiencies in HEK293 cells compared to other subtypes as well as to shRNA-mediated and CRISPRi knock-downs [192]. In CHO cells, targeting mRNA by transient CasRx expression resulted in up to 80% knock-down of various common cell line engineering targets [193] and might be, especially in comparison to transient CRISPRi, a promising alternative for temporary gene knock-down. Experiments performed in our lab, confirm the functionality and efficacy of these tools (unpublished data). Similar to the dCas9-based synTFs described in 0, also catalytically inactive Cas13 variants have been developed and fused to effector domains that can be repurposed for RNA editing [194–196] or mapping of RNA-protein interactions [197–199]. These novel applications have not yet many applications in CHO cell research, but the versatility of these tools and the constant improvement make this technology very appealing for novel cell engineering approaches.

Considerations for cell engineering

Post-transcriptional regulation tools allow the control of gene expression levels largely independently from the transcription rate. Dynamically controlling mRNA levels post-transcriptionally also enables a quicker response to extrinsic signals, as the step of transcriptional initiation is omitted and the transcripts are already available [179]. RNAi-based tools offer many advantages to control gene expression levels, as (i) they can easily be adapted to various targets by simply changing their complementary sequences, and (ii) systems are available, e.g. by employing transcriptional expression tools that control expression of the siRNA or miRNA itself, and allow tight control of the target gene expression. Still, as with all nucleic acid-based regulation tools that rely on complementary base-pair binding, off-targets (unintentional regulation of other non-target genes) can be an issue and must be evaluated before use. Similar considerations hold for CRISPR/Cas13 systems. They can be adapted to basically knock-down any endogenous gene. On top, they can be used for advanced tasks such as RNA editing, and thus open up new possibilities to engineer mammalian cells. As with synTFs discussed in the previous section, these systems require

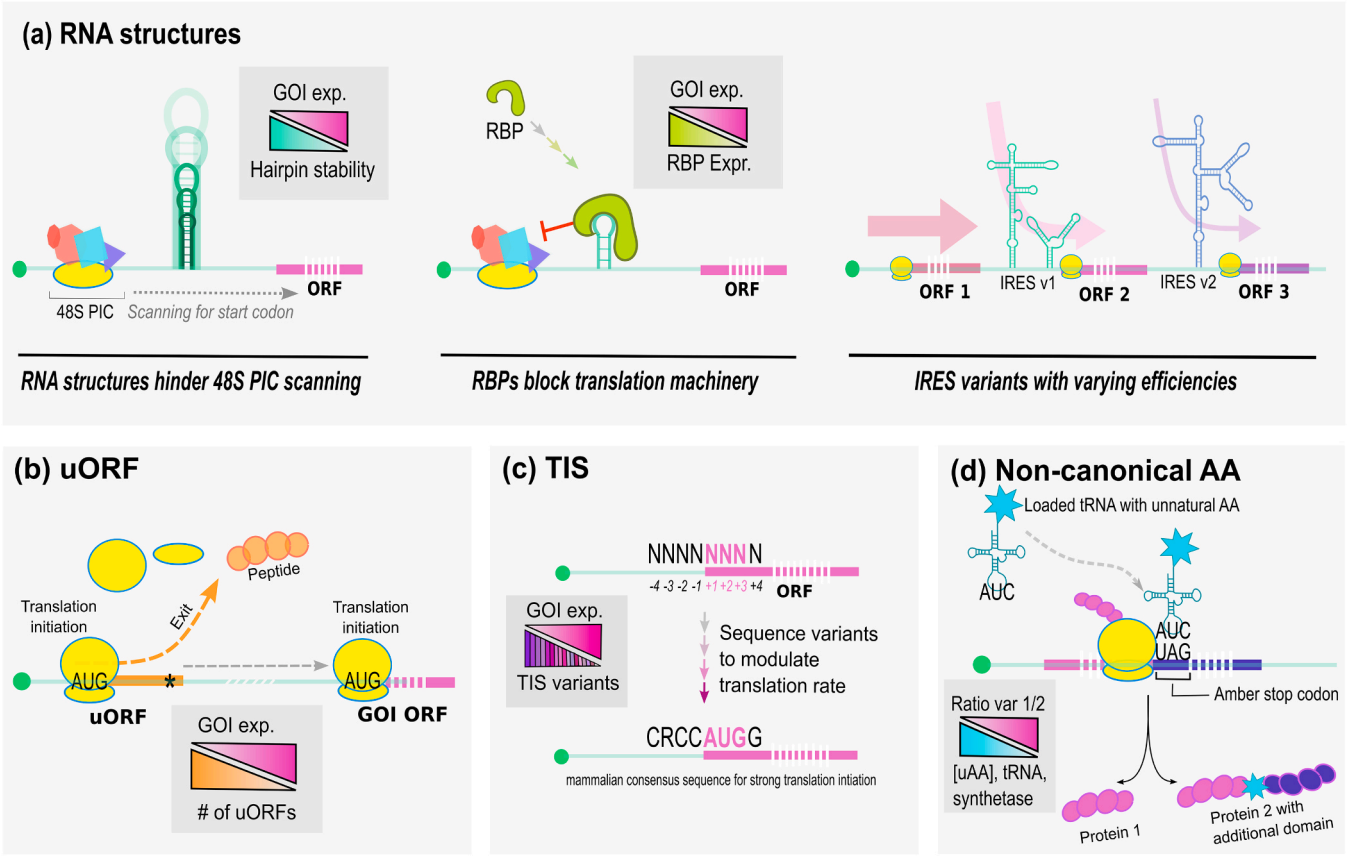

Fig. 4. Translational regulation toolboxes. (a) RNA secondary structure elements. ORF = open reading frame. Left: RNA structures in the 5`-UTR that interfere with scanning of the 48S pre-initiation complex (PIC). Middle: RNA structure(s) in the 5`-UTR can attract RNA-binding proteins (RBP) that interfere with the translational machinery. Right: Including more than one ORF in the transcript. Translation of the second or even third ORF could be initiated by IRESs. Naturally occurring or mutant libraries of IRES variants can induce translation differentially strong (indicated by varying arrow size). (b) Upstream ORFs (uORFs) located in the 5`-UTR initiate premature translation. Some ribosomal subunits remain bound to the transcript and resume scanning and eventually reinitiate translation of the desired ORF. (c) Translation initiation sequence (TIS) can be randomly mutated to obtain varying translation rates. (d) Non-canonical or unnatural amino acids (AA) can be used to control different variants of protein.

heterologous expression of the Cas13 and the guiding RNA in the cell line of choice. Consequently, pre-engineering of the used cell lines is required and in addition cellular resources might be drawn for the recombinant production of the Cas13 protein that can lower the titer of the actual recombinant product if used in a stable set-up.

RNA-based gene switches offer very good dynamic control of gene expression. Multiple structures that are responsive to distinct types of small chemicals are available. Therefore, these molecular toolboxes can effectively be employed to control the expression levels of multiple genes simultaneously. On the downside, activation and repression of gene expression with such structures require the presence of small chemicals that might be cytotoxic, e.g. theophylline [200], and are therefore not ideal for use with cells in the production process of a biopharmaceutical product, or they might impact other aspects of the cellular phenotype in other ways. In addition, such structures are not easily applied to endogenous genes and they may be expensive on a larger scale.

Overall, post-transcriptional regulation tools offer highly dynamic control of multiple gene expressions and will likely be an important part in the implementation of complex genetic circuits that require multi-level control of gene expression levels in response to extrinsic or intrinsic signals.

Translational regulation

Translation of mRNA sequences into proteins is a crucial process in cellular organisms and required by all forms of life. Unsurprisingly, the translational mechanisms are highly conserved among different species [201]. From this perspective, developing toolboxes that facilitate precise regulation of translational rates would support the use of such a tool in various species without major adjustments and (re-)investigation of the tool's functionality (Fig. 4).

RNA structures

Nascent RNA molecules self-assemble into secondary and tertiary structures. The most prominent examples of highly structured RNAs are likely the transfer (t)- and ribosomal RNAs. But also, mRNAs or other classes of non-coding RNAs, such as the miRNA precursors or lncRNAs, form secondary and tertiary structures that are required for certain functions, such as RNA processing and regulation. Alongside the mRNA sequence, regulatory RNA structures can be found predominantly in the 5`- and 3`-UTRs. The structures range from simple "hairpin" also known as "stem-loop" structures, to complex pseudoknots or RNA G-quadruplexes, or metabolite-sensitive riboswitches such as aptazymes. While some of these structures interfere with the formation of mRNA loops, the assembly of the translation machinery at the 5`-cap or slow down the speed of the translation machinery, other structures affect the accessibility of e.g. start codons or splice sites and therefore the translation progress. For more detailed information on the biological roles of 5`- and 3`-UTR structures the reader is referred to reviews of Leppek at al. [202] or Mayya and Duchaine [203]. In this part we will focus on how RNA structures can be repurposed to control protein expression levels (Fig. 4a).

RNA structures in the 5´-UTR, such as RNA hairpins, were already described in the late 1980's to be inhibiting the ribosome scanning and consequently the translation of downstream encoded genes [204,205]. Babendure and colleagues then characterized 5`-UTR RNA hairpins in various mammalian cell lines based on their thermodynamic stability (minimum free energy or "MFE" (ΔG)), the GC content and their relative position to the 5`-cap and thus provided a basis for the intentional use of such structures to control gene expression levels [206]. Eventually, RNA hairpin structures were set-up as a toolbox that can be employed systematically to tune protein expression levels in mammalian cells [28]. In this study RNA hairpins were employed to balance expression levels of an IgG heavy chain (HC) and light chain (LC) to ultimately improve the antibody titer and quality. Importantly, these findings not only demonstrated the application of RNA hairpins in CHO cells, but also show that they function very similarly in other, widely used mammalian expression systems for biopharmaceutical production, such as HEK293 cells. Intriguingly, in comparison to studies in yeast [207,208], the RNA hairpins show very similar functionality. These observations support the rationale of developing molecular tools that impact the translational process, to establish a "universally" applicable toolbox for engineering of mammalian or rather, eukaryotic systems in general.

RNA structures can also function as an entry point for the translational machinery on an mRNA and thus facilitate cap-independent translation. These structures are known as "internal ribosome entry sites" or short "IRES" and were first identified in viral RNA genomes [209,210], but were later also found in eukaryotic RNA molecules such as circular RNAs [211]. Most importantly, repurposing IRES for molecular biology enabled the expression of two distinct proteins from one mRNA strand. Eventually, this allowed to couple translation of two, or even more factors, from a single transcript. Because of this, researchers could put multiple genes under the control of a single promoter, which is classically not possible in eukaryotic cells, but only in prokaryotes. Different IRES variants were discovered that mediate translational initiation with different efficiencies and thus allow to establish various gene expression levels [209,212–214]. Varying and mutating the sequences of a particular IRES is another way that was described to alter translational levels of the downstream encoded gene [215,216]. Koh and colleagues utilized an IRES library to investigate IgG HC to LC expression ratios that are translated from the same mRNA transcript in CHO cells. The authors also demonstrated that different IRES variants function similarly across various mammalian cell lines, such as CHO, HEK293, BHK, 3T3 and COS7. Intriguingly, however, sequence mutations were introduced predominantly in the AUG start codons that are present in the IRES sequence [215]. This suggests that translational regulation in this case is actually achieved by modulation of translational initiation rather than by an altered secondary or tertiary structure of the mRNA.

Upstream open reading frames

Open reading frames (ORF) upstream of the actual protein-coding sequence on the transcribed gene, so-called "uORFs", are regularly found in mammalian mRNAs (Fig. 4b). These short sequences encode for small peptides, which have a start and stop codon, and are considered to be an internal expression regulation mechanism that governs the expression of the downstream situated ORF of the target gene. uORFs are involved in many crucial cellular mechanisms, such as stress response or mRNA decay, where their presence regulates expression of e.g. the stress response factors, such as ATF4 (reviewed in [217]). Upon encounter with an uORF, the ribosome can either (i) translate the uORF, stall and mediate mRNA decay, (ii) translate the uORF and reinitiate at the actual ORF or (iii) scan through the uORF and initiate translation at the actual ORF [218,219]. The expression level of the downstream encoded target genes therefore depends on i) how efficiently the uORF is translated (uAUG context, length, position of the stop codon) and ii) how efficiently translation is reinitiated at the actual ORF (48S subunit stays associated with mRNA and resumes scanning until it identifies a new start site) [217]. Noteworthy, not all upstream start codons initiate translation as this not only depends on the presence of the AUG start codon, but also on the adjacent bases (the so-called translation initiation sequence (TIS) (discussed in 0) or RNA secondary structures [220].

Ferreira and colleagues designed synthetic 5`-UTRs with up to three uORFs that encode for two amino acids and in addition varied the TIS around the start codons to impact the expression levels of the target gene. The authors screened their constructs in CHO-K1 among multiple other mammalian cell lines, and eventually could cover the recombinant expression range from ~ 0–1-fold in multiple steps. Interestingly, they found that these uORFs constructs function very similarly across all tested mammalian cell types, and therefore also support the use of translation impacting tools as generally applicable engineering tools to

control gene expression levels in mammalian cells. Moreover, the authors also employed their technology to control the cell cycle in CHO cells by tuning the expression of p21 [221]. Recently, Jones et al. described an endoRNase-based feedforward system that allows robust gene expression and performance of genetic devices despite variable cellular resources, e.g. transcriptional and translational, in multiple mammalian cell lines. Part of their toolbox is based on varying numbers of uORFs (up to twelve, as previously characterized [222]) that regulate the translation of the endoRNase which controls output levels of the transgene and eventually decouples output signals from cellular resources [223]. This study of Jones and colleagues nicely demonstrates how multiple methodologies to control gene expression levels from exogenously constructed genetic devices can be employed to allow robust and predictable gene expression in mammalian cells.

Translation initiation sequence and signal peptides

Translation of the mRNA sequence into an amino acid sequence is initiated at the start codon AUG. However, the efficiency and corresponding rates of translation are not solely dependent on the start codon itself, but also on the Kozak sequence surrounding the AUG triplet. In mammalian cells the consensus sequence is 5`- CRCCAUGG − 3` for a strong initiation of translation [224,225]. Intriguingly, Lee and colleagues mapped TISs in mammalian cells and found that only around half of the identified TISs contain AUG as a start codon, whereas the other half contains start codons in the form of CUG or AUG-variants (e.g. UUG, GUG). Additionally they found that almost 50% of the transcripts harbor more than one TIS (upstream or downstream TIS), highlighting the importance of translation of alternative ORFs, such as uORFs, in cellular systems [226].

TIS variants have been used in multiple studies to alter translational rates in mammalian cells (see Table 1) (Fig. 4c). As already described in the previous section, Ferreira et al. employed TIS variants in combination with uORFs in a plethora of mammalian cell lines to control transgene expression levels [221]. Petersen and colleagues used TIS variants in CHO cells and found comparable regulation efficiencies of the tested elements as in yeast systems [227]. A recent study by Blanco et al. described a library screening of Kozak sequence variants in CHO cells and could identify versions that enable stronger expression compared to the wildtype Kozak sequence [10]. On the other hand, Baumann et al. rationally altered the start codon AUG to CUG and to UUG, respectively, in a CD4 reporter protein. Initially thought as a strategy to select cells, i.e. by fluorescence activated cell sorting, that demonstrate high expression capabilities for the recombinant protein, it later turned out that stringent isolation of highly productive cells rather selects cells that re-mutated their alternate start codon back to AUG [228]. This finding highlights that mutations within TIS variants can easily occur and eventually could guide the expression strength of the regulated gene towards an unexpected direction.

Signal peptides (SP) impact the translation and secretion of proteins and can be repurposed for post-translational regulation. SPs are short 5–30 amino acid residues commonly present at the N-terminus of a protein and ensure its proper secretion, folding and 3D structure. The role of an SP is to ensure the binding of the newly translated protein chain by the signal recognition particle in the cytosol, mediating the rate of translation itself and targeting (docking) the ribosome-nascent protein complex into the ER lumen [229,230]. SPs therefore function not solely post-translationally but can also regulate the rate of translation itself. The function of specific SPs is strongly preserved among evolutionary different species [231,232], yet the primary sequence differs greatly even among similar proteins [233,234]. To improve recombinant protein production in mammalian cells, the genetic sequence of a recombinant gene may be engineered to ensure secretion of proteins with either no signal sequences, the original signal sequence of the protein may be substituted to improve secretion in a certain host or a signal sequence may be chosen through a screening approach to improve specific productivity [235–240]. These optimization strategies have been demonstrated in many instances to improve recombinant productivity in CHO cells [235,241–247] [237–239,248]. Taken together, SPs can have a major impact on the secretion efficiency of the target protein and should therefore be considered when designing expression cassettes of secreted proteins.

Non-canonical amino acids

Other mechanisms, such as the incorporation of non-canonical (also known as unnatural) amino acids (ncAA or uAA), have been described to control translation (Fig. 4d). Non-canonical amino acids are built into the growing peptide chain at positions that usually mediate translational termination, the so-called “amber” stop codon that corresponds to the UAG triplet. To do so, it is required to provide three things to the cellular system: the non-canonical amino acid, the orthogonal tRNA that recognizes the amber stop codon and an orthogonal synthetase that loads the uAA on this respective tRNA. Further, a gene construct is required where the codon for the amino acid to be replaced by the non-canonical amino acid is accordingly altered. Consequently, controlling the availability of one (or multiple) of these parts allows to control the incorporation of the unnatural amino acid into the protein-of-interest. Thereby, depending on where in the coding sequence the artificial amber stop codon is placed, it becomes possible to control the expression of the protein-of-interest [249]. This could for instance be used to generate and study function of protein domains (if the amber codon is placed upfront of the domain of interest to mitigate its expression) or to induce the general expression of the protein-of-interest (if the amber stop codon is placed before the actual codon sequence). For a more detailed review of uses of non-canonical amino acids in mammalian systems the reader is referred to the review of Nödling et al. [250].

uAA are also widely employed in the manufacture of antibody drug conjugates (ADCs). ADCs depend on the chemical ligation of a drug molecule to the manufactured protein. Therefore, due to the vast number of uAA available, they are well suited to facilitate the site-specific conjugation of drug molecules to an antibody [251]. CHO cells have been established as a production system for ADCs by incorporation of uAA [252–255]. Roy et al. installed a selection system to screen for efficient amber suppression using a dual fluorescent reporter construct with an amber stop codon in between the coding sequences. Eventually, selected CHO cells with efficient amber suppression were subjected to stable IgG production (IgG contained an amber in the variable region) which resulted in reduced screening scale necessary to identify efficient production cell lines [254].

Considerations for cell engineering

Molecular toolboxes that regulate gene expression levels at translation have demonstrated to facilitate fine-tuning of transgene abundance levels. Interestingly, all of the here introduced tools were found to be functioning in very similar ways in multiple mammalian host cell lines and also in yeast. This, as stated in the short introduction to this subchapter, can likely be attributed to the high conservation of the translation process across species [201]. In this regard, tools that regulate translational strength of the GOI could be (quickly) adapted and used without major characterization studies in mammalian host cell lines other than the cell type in which they were established. This could, for instance, be advantageous when novel, non-model expression host cell lines are established for which no or very limited information, understanding or engineering tools are available [256].

Another benefit of the introduced translational regulation toolboxes is that, except for the IRES variants, they are all small (e.g. ~90 bp for the strongest RNA hairpins elements [28,206], down to 6 bp for the hexamers of the TIS variants [227]). These small sized elements enable efficient expression strength control without substantially altering the size of the genetic construct. This is of particular importance in constructs where small sizes are desirable or even required due to limited space availability, as for instance in AAV vectors used in gene therapy. Translational rates are determined by a short sequence stretch and the

individual bases have tremendous impact on the strength of translation initiation. Consequently, (randomly) occurring base mutations, as for instance reported in Baumann et al. [228], might alter the translation profile of the GOI substantially. This, of course, applies for all sequence-based toolboxes, but might be of special importance in TIS variants due to their comparatively small sizes.

The special advantage of incorporating non-canonical amino acids is that they can be employed as an inducible system, e.g. by supplementing the non-canonical amino acid when the protein-of-interest should be fully expressed. Its specific advantage over inducible promoters is that it does not require extra transcription and mRNA export, but can affect expression levels of the target protein relatively quickly [250]. Although the toolboxes usually rely on the incorporation at artificially introduced UAG amber stop codons, that are the least frequently prevalent stop codons in mammalian cells, off-targets, meaning here incorporation of the unnatural amino acid in endogenous targets, might be possible and need to be considered [257].

Likely the biggest disadvantage of the here described toolboxes is the difficulty to apply them for expression level regulation of endogenous genes, as all of them rely on the integration of specific elements into the vector constructs. Contrary to options like inducible promoters or aptazymes, where a single element that responds to exogenously controlled concentrations of a small inducer molecule regulates the expression level of the GOI, various elements are required with the debated translational regulation toolboxes. Thus, once introduced into the genetic cassette and transferred to the system, the expression strength cannot easily be impacted and dynamically regulated. Therefore, such tools are likely most beneficially employed in settings where GOI expression rates should be tuned to a defined and fixed level and not in circumstances in which dynamic regulation of the GOI expression strength might be desired.

Lastly, a specific consideration is addressed to tools relying on uORFs. As with these toolboxes small peptides are formed alongside the expressed protein, it could potentially be stressful for the host cell line to cope with the (potentially) abundant presence of these peptides. Additionally, the cells might need to degrade them again to supply sufficient amino acids for the remaining proteins in the cells. From the viewpoint of optimizing the usage of cellular resources to free up as many of these resources as possible and channel them into the recombinant manufacturing of the target product, it might therefore be counterintuitive to install uORFs in engineering approaches where recombinant titers should be elevated. This would likely result in higher consumption of resources for translational initiation, elongation, termination, peptide degradation and reloading to tRNAs with amino acids that might be missing for the formation of the actual recombinant product.

Translational regulation tools therefore represent valid options to control and screen for various expression levels of an introduced transgene but are less likely to be employed in engineering scenarios that require dynamic changes of the gene expression levels or modulation of endogenous genes. Nonetheless, their small size and functionality within the framework of an evolutionary conserved mechanism supports the use of such tools in various mammalian cell types, maybe even in non-model and other eukaryotic systems (that lack established toolboxes) without constraining size limitations of the genetic constructs.

Post-translational regulation

Controlling the expression levels after a protein is translated is a common mechanism for cellular systems, for instance regulating the activity of a protein by post-translational modifications, such as phosphorylation of serine, threonine or tyrosine residues. Phosphorylation can induce rapid conformational changes of the protein structure, thereby enabling the cell to specifically control the activity of the modified factor, and thus has a prominent role in signaling cascades [258]. Other naturally occurring mechanisms control protein expression by specifically marking it for degradation in the proteasome, e.g. through ubiquitylation [259]. Also, synthetic tools to control protein expression levels in a tunable manner were developed.

Protein degradation systems (degrons)

Protein degradation tags, or short degrons, are protein elements that induce degradation through cell internal protein degradation pathways. By fusion of the protein-of-interest (POI) with an inducible degron, potent and tunable protein degradation is possible. The auxin-inducible degron (AID) system, a system that is naturally occurring in plant cells, has been adapted to mammalian cell lines (including CHO-K1) and even yeast [68,260,261]. In the presence of the hormone auxin, the endogenous E3 ubiquitin ligase is guided to a POI fused to an AID domain, marking it for degradation via the proteasome. This system allows quick, reversible and tunable control over protein expression levels in mammalian cells [260]. A recent publication investigated the use of a tunable degron-based CRISPR-Cas toolkit [262] to fine-tune the abundance of Cas9 molecules and through this the expression of endogenous genes. Fusion of the degradation Tag (dTAG)− 13 inducible $FKBP12^{F36V}$ degron domain to CRISPR-dCas9 (0) or CasRx (0) allowed to regulate – via dTAG-13 titration - the expression levels or mRNA abundance of target genes.

Protein splicing

Another strategy to control protein expression was reported by employing the mechanism of protein splicing to control the fusion of the protein-of-interest [263,264]. To this end, the POI is split into two so-called exteins (N- and C-terminal part of the protein-of-interest) that are fused to split inteins and variable binding domains. Upon induction of binding the two binding domains, e.g. mediated through a protein scaffold that contains binding sites for each of the binding domains, the inteins are brought in close proximity to one another and consequently induce splicing and therefore fusion of the two POI exteins. Eventually, this facilitates the generation of a functional POI in a controlled manner [264].

Considerations for cell engineering

Post-translational tools to regulate protein expression levels have the advantage that they act on the last possible stage of the biosynthesis of a protein. Therefore, they enable very fast responses to external signals, contrary to e.g. regulating transcription where mRNAs first have to be generated, processed and eventually translated. Unlike transcription, where all of the downstream regulation mechanisms might impact the ultimate expression level of a gene, interfering at the post-translational stage is beneficial as fewer regulation points (e.g. secretion might be one) can impact the final expression level. Consequently, regulation at this stage might be preferred if fast and direct responses are desired. Intriguingly, studies have highlighted that the functionality of post-translational regulation tools can be improved by combining it with transcriptional regulation mechanisms [265,266].

On the other side, post-translational regulation tools in many cases require in-advance engineering of a host cell line by introducing additional genes that enable functionality of the system, e.g the AID system (s). In addition, fusion proteins of the POI and the (de-)stabilizing domain must be established. This, on the one hand might limit using such systems to balance subunits of a multimeric recombinant protein, as the extra domain could interfere with the assembly and/or functionality of the product. On the other hand, it is difficult to install such toolboxes to control expression levels of endogenous genes, especially in cellular systems where homologous-directed repair is inefficient (making it difficult to install the required domains at precise positions in the genome) [267]. Furthermore, controlling the expression level(s) by expressing proteins to eventually induce their degradation seems counterintuitive from the rationale and aim of efficient usage of cellular resources.

For the same reasons, it might be counterintuitive to control the expression by interfering with processing of proteins as these

mechanisms could trigger cellular stress responses, e.g. the UPR pathway. Also, engineering factors that are not secreted to or located in cellular compartments in the secretion pathway (ER, Golgi,…), but stay in the cytosol or are even located in the nucleus (most importantly transcription factors) could not be engineered with such a toolbox.

Genetic circuits

Eventually, the herein discussed technologies will aid in establishing dedicated genetic systems that enable rational regulation of mammalian cell lines. So-called "genetic circuits" refer to introduced transgenes that respond to specific input signals by altered expression levels that can be controlled on various layers. So far, circuits are distinguished as DNA- or RNA-based or a mixture of both. All have in common that these genetic constructions usually require inducibility of the regulated genes. Through the combination of various inducible systems, Boolean logic gates can be established that control the expression of the transgene(s). Common logic gates are for instance YES, NOT, AND, OR; NOR or NAND gates [61]. These gates translate the input signals, usually small chemical ligands such as doxycycline or theophylline, into a genotypic and eventually a phenotypic response of the cellular system. Consequently, cellular systems thereby become (to a small degree) computable in the sense that phenotypic traits can be controlled through extrinsic signals controlled by the human user [268]. In mammalian cell lines, genetic circuits have been established mostly in the context of engineering cell-based in-vivo applications (reviewed in [6269]). Engineering genetic circuits into mammalian cell factories, however, has so far only found limited applications. An intriguing example of how genetic circuits can be employed to enhance the production of therapeutic proteins was described by Chang and colleagues, who employed a genetic module of two genes to dynamically established N-glycan variants [60]. Ausländer et al. reported the use of a three-input AND logic gate in HEK293 cells to control expression of a recombinant SEAP [189]. Recently, Zeh et al. used hypoxia responsive elements to drive transgene expression in response to oxygen limitation [62]. Although the number of reported cases in which multi-gene genetic circuits were successfully employed to enhance recombinant therapeutic production in mammalian systems is currently still humble, we strongly believe that future engineering strategies will move towards this direction. Genetic circuits could advantageously be used to control phenotypic traits of mammalian cell lines in a biopharmaceutical production run, e.g. by installing responsive anti-apoptotic genetic circuits that are activated towards the end of a production process. Thinking further ahead, it could be even more beneficial when such a regulatory mechanism is installed that does not require interferences from the exterior but is able to self-regulate expression of the desired genes in response to certain intrinsic signals, as intriguingly described by Lin et al. [270].

Conclusion

With the increasing understanding of mammalian expression systems, generated through the collection and analysis of multiple -omics data sets, strategies to optimize and eventually gain rational control over the cellular phenotype can be developed. Likely, more demanding engineering strategies need to be evolved to gain phenotypic control that goes beyond "simple" overexpression or knock-out of a single genetic factor. Rather, a balanced expression of the recombinant product itself in addition to regulated expression strengths of various engineering factors or even the installment of extrinsically controllable genetic circuits is required. These novel approaches will consequently require dedicated molecular tools that facilitate their successful translation into the biological system and allow for combinations that match each individual gene's specific requirement. This review highlights that an impressive number of tools to regulate gene expression levels of multiple genes in various mammalian expression systems have already been developed and provided to the research community. Also, many of these toolboxes were applied to improve recombinant protein manufacture in CHO cells and other mammalian cell systems. Ultimately, we strongly believe that these developments in the field of system and synthetic biology will find exciting applications and will further enhance mammalian systems as cell factories for the efficient high quality production of complex therapeutic products.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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