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Stable mammalian producer cell lines for structural biology

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The mammalian cell lines HEK293 and CHO have become important expression hosts in structural biology. Generating stable mammalian cell lines remains essential for studying the function and structure of recombinant proteins, despite the emergence of highly efficient transient transfection protocols. Production with stable cell lines can be scaled up easily and high volumetric product yield can be achieved. Protein structure reports of the past two years that used stable cell lines were surveyed for this review. Well-established techniques and novel approaches for generating stable cell lines and stable cell pools are presented, including cell sorting, site-specific recombination, transposons, the Lentivirus system and phage integrases. Host cell line optimization by endoglycosidase overexpression and sequence-specific genome engineering is highlighted.

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Introduction

Generating pure, soluble and homogeneous protein is a major step in the overall process of protein structure determination. The choice of the expression system has a great influence on the quality and quantity of the produced recombinant protein. The Human Embryonic Kidney cell line HEK293 and the Chinese Hamster Ovary cell line CHO are excellent host cells for robust secretion of mammalian proteins with appropriate post-translational modifications [1]. These cell lines are used for production of secreted mammalian and viral proteins and soluble ectodomains of transmembrane proteins, but also for complete membrane proteins (Table 1). Cytosolic proteins and complexes can be produced with stable mammalian cell lines, but the yield is usually low compared with other expression systems. The long-awaited crystal structure of the cytosolic mTORC complex was obtained upon overexpression of its subunits in a stable HEK293 cell line [2••].

For recombinant protein overexpression, an expression vector for the protein of interest is transferred into the cell's nucleus by transfection. In a transient transfection experiment, the protein of interest is harvested a few days later. Alternatively, a stable cell line is generated from transfected cells that have integrated the vector into their genome. Stable cell lines overexpress the target protein uniformly and indefinitely. Protein production with a stable cell line is therefore reproducible and can be scaled up easily. Recent protein structure reports using HEK293 or CHO cell lines were surveyed for this review and it was found that transient transfection and stable cell lines were used with around the same frequency. New and improved technologies for generating stable cell lines are expected to increase their use in the future.

Establishing stable cell lines requires substantial time and effort in comparison to transient transfection processes. An expression vector with the gene of interest has to be inserted into the host cell genome. Using standard methods, the efficiency of genome integration is low. Moreover, only very few cells will integrate the vector into a highly transcribed region and will produce sufficient amounts of recombinant protein. Even then, transgene expression is often silenced upon long term cell culture. Isolating and characterizing a large number of clones is therefore required, which can take several months of laboratory work. Fortunately, stable cell line technology is improving rapidly on the levels of host cell line, integration process and selection of high-producer cells.

Crystal structures of proteins produced by stable cell lines reported during the past two years are listed in Table 1. The table provides an overview of the host cell lines and experimental techniques currently used in structural biology. The most common approach involves transfection with vectors carrying a selectable marker for random chromosomal integration, followed by isolation and screening of single cell clones. This process was reviewed in three recent publications [3,4,5] and protocols for establishing stable cell lines for structural biology with antibiotic selection markers have been published [6,7]. The performance of the antibiotics hygromycin B, neomycin, puromycin and Zeocin as selection markers for stable cell line development was recently compared [8].

Table 1

The Protein Data Bank (PDB) was searched for mammalian protein structures, excluding antibodies, that were released later than July 2012. Selected proteins that had been produced by stable cell lines are listed

	Crystallized protein	Host cell line[a]	Vector	Stable cell line generation[b]	PDB entry and Ref.
Cytosolic proteins	mTOR:LST8 complex	HEK293-F	pcDNA 3.1 (+)	Co-expression of mTORC subunits using different antibiotics	PDB: 4JSN [2••]
Membrane proteins	Rhodopsin	HEK293 GnTI$^-$			PDB: 4BEY [54]
	GABAA receptor	HEK293S GnTI$^-$			PDB: 4COF [55]
	5-HT3A receptor	T-REx-293	pcDNA5/TO		PDB: 4PIR [56]
Secreted proteins	Prothrombin	BHK	pNUT	Lipofection, MTX	PDB: 4HZH [57]
	Aminopeptidase N	CHO Lec3.2.8.1	pBJ5-GS	MSX	PDB: 4F5C [22]
	Integrin α_4	CHO Lec3.2.8.1	pcDNA3.1/Hygro		PDB: 4IRZ [58]
	MHC class II HLA-DQ1: antigen:TCR complex	CHO Lec3.2.8.1	pEE13.1	MSX	PDB: 4GRL [21]
	CD200 receptor	CHO Lec3.2.8.1	pEE14		PDB: 4BFE [59]
	DC-LAMP	CHO Lec3.2.8.1	pFS-sigHis	Electroporation, RMCE, G418	PDB: 4AKM [39•]
	Neuroligin-1	CHO Lec3.2.8.1	pSGHV0	Electroporation, puromycin	PDB: 3VKF [60]
	HLA-DM	CHO Lec3.2.8.1			PDB: 4FQX [61]
	ICAM-5	CHO Lec3.2.8.1		MSX	PDB: 4OI9 [20]
	Tyrosine kinase receptor RET	CHO Lec8	pcDNA3	Lipofection (Effectene), G418	PDB: 4UX8 [62]
	Insulin receptor	CHO Lec8	pEE14	Lipofection (Lipofectamine 2000), MSX	PDB: 3W11 [23]
	Cholesteryl ester transfer protein	CHO DG44			PDB: 4F2A [63]
	Receptor tyrosine-kinase erbB-4	CHO Lec1	pSGHV0	Lipofection (Fugene), pcDNA3.1 co-transfection, G418	PDB: 3U7U [64]
	Folate receptor α	CHO duk$^-$ (kifu.)	pSGHV0	Electroporation, MTX	PDB: 4KM6 [14]
	Acetylcholinesterase	CHO-K1	pGS	jetPEI, MSX	PDB: 4BDT [24]
	LIMP-2	HEK293S GnTI$^-$	pNeoSec	ΦC31 integrase (stable cell pool)	PDB: 4Q4B [42]
	Glutamate receptor 4	HEK293 GnTI$^-$	pHLsec		PDB: 4GPA [65]
	Tumor antigen 5T4/WAIF1	HEK293 GnTI$^-$	pURD	ΦC31 integrase, puromycin	PDB: 4CNC [48]
	Transcobalamin-1	HEK293 GnTI$^-$		Lipofection (lipofectamine)	PDB: 4KKI [66]
	Integrin $\alpha_X\beta_2$ heterodimer	HEK293S GnTI$^-$	ET1 (pIRES2-EGFP), pEF1-puro	Ca-phosphate, FACS, puromycin, G418	PDB: 4NEH [29]
	von Willebrand factor	HEK293S GnTI$^-$	ET8	PEI, G418	PDB: 4NT5 [67]
	Aminopeptidase N	HEK293S GnTI$^-$			PDB: 4FYQ [68]
	NPP1 pyrophosphatase	HEK293S GnTI$^-$			PDB: 4B56 [69]
	Angiopoietin-2	HEK293	pcDNA3.1 hygro	Lipofection	PDB: 4JZC [70]
	Netrin-1:neogenin complex	HEK293	pcDNA3.1+		PDB: 4PLN [71]
	RNaseT2	HEK293 (kifu.)	pcDNA3.1/myc-His	Lipofection (Effectene)	PDB: 3T0O [15]
	TGF-β-receptor-3	HEK293 EBNA		Lipofection (Lipofectamin 2000), puromycin	PDB: 4AJV [72]
	Folate receptor α	HEK293	pcDNA6	blasticidin	PDB: 4LRH [73]
	IgG receptor FcRn subunit p51	HEK293-6E	pLVX	Lentivirus	PDB: 4K71 [46]
	Nectin1, 2, 3, Necl-5.	HEK293-F	pCEP4	Lipofection (Lipofectamin 2000), hygromycin B	PDB: 4FMF [74]
	Monocyte differentiation antigen CD14	HEK293-F	pDisplay	G418	PDB: 4GLP [75]
	Acetylcholinesterase	HEK293-H	pJT1 Fast Dest	Lipofection (Lipofectamin 2000), ΦC31, hygromycin B	PDB: 4EY4 [49]
	Platelet glycoprotein Ib	HEK293T	ET-6		PDB: 4C2A [76]
	Galactocerebrosidase	HEK293T	pSecTag2B		PDB: 4CCC [77]
	O-fucosyltransferase 2	HEK293T (kifu.)	pSecTagB	jetPEI, zeocin	PDB: 4AP5 [13]

[a] kifu., the GnTI inhibitor kifunensine was used during protein production.

[b] Cells were transfected by lipofection, electroporation or with polyethylenimine (PEI, jetPEI). Recombinant cells were selected with antibiotics (blasticidin, G418, hygromycin B, puromycin, Zeocin), with the glutamine synthase inhibitor methionine sulfoximine (MSX) or with the DHFR inhibitor methotrexate (MTX). FACS, GFP-positive cells were isolated by cell sorting.

Engineering of host cell lines for stable cell line generation

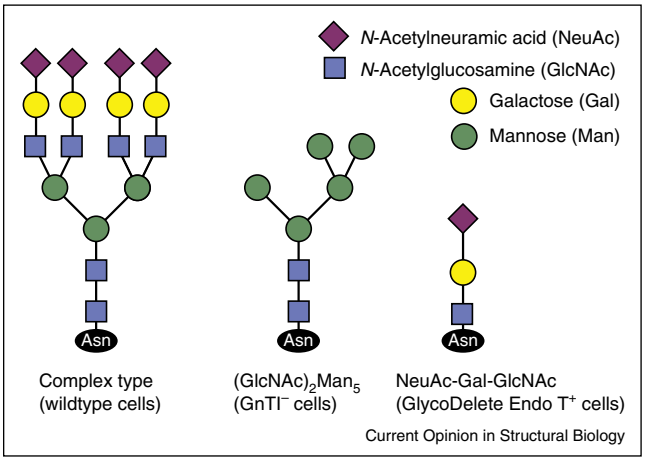
Glycosylation-deficient cell lines

Most secreted mammalian proteins are glycosylated, which can interfere with crystallization [9••]. Glycosylation sites that are not required for folding or secretion are therefore removed by mutagenesis [9••]. The processing of N-linked glycans from the high-mannose type to the larger, complex type requires the enzyme N-acetylglucosaminyl-transferase I (GnTI, MGAT1) (Figure 1). The GnTI-deficient host cell lines HEK293S GnTI$^-$ and CHO Lec3.2.8.1 produce glycoproteins with high-mannose type glycans. These glycans can be readily trimmed further to a single GlcNAc sugar unit by endoglycosidase treatment [10,11]. The HEK293S GnTI$^-$ cell line [10] (ATCC CRL-3022) is currently the most popular cell line in structural biology (Table 1). It is used for transient and stable expression. High-mannose type glycosylation is also obtained by cultivation with the GnTI inhibitor kifunensine [12]. Kifunensine was applied for crystal structure determination of the glycoproteins O-fucosyltransferase [13], folate receptor α [14] and RNaseT2 [15] (Table 1).

Intracellular endoglycosidase overexpression

Recently the 'GlycoDelete' HEK293 cell line was developed that carries a heterologous endoglycosidase in its Golgi apparatus [16••]. This resulted in robust secretion of deglycosylated glycoproteins (Figure 1). The GlycoDelete study demonstrates that protein deglycosylation is possible in the Golgi apparatus, where glycoproteins traveling the secretory pathway have already passed the quality control in the endoplasmic reticulum. Cells that secrete glycoproteins with minimal glycosylation would enable crystallization without further *in vitro* deglycosylation.

Figure 1



N-linked sugar classes produced by mammalian cell lines. Complex-type glycans are synthesized as a heterogeneous mixture of bi-antennary, tri-antennary and tetraantennary forms. Lack of N-acetylglucosaminyl-transferase I (GnTI) prevents the formation of complex sugars and results in high-mannose type protein glycosylation. High-mannose sugars consist of a variable number of mannose residues attached to a chitobiose ($GlcNAc_2$) core. Overexpression of endoglycosidase T (Endo T) in the Golgi apparatus of the GnTI$^-$ 'GlycoDelete' HEK293 cell line leads to intracellular cleavage of N-linked sugars after the first GlcNAc residue. The cleavage product is partly modified with galactose and N-acetylneuramic acid residues.

Sequence-specific genome engineering

The cell lines CHO Lec3.2.8.1 and HEK293S GnTI$^-$ were created by chemical mutagenesis, followed by selection for glycosylation deficiency. Chemical mutagenesis lacks specificity, leading to random mutations throughout the genome. Sequence-specific genome engineering represents an elegant alternative that greatly reduces unwanted mutations. Meganucleases with long recognition sequences cleave genomic DNA at rare sites and can be used to introduce gene-inactivating mutations more specifically [17]. Nucleases linked to programmable, sequence-specific DNA-binding modules, such as zinc finger nucleases (ZFN), TALE nucleases and the CRISPR/Cas9 nucleases allow for modification of arbitrary genetic loci with excellent specificity [18]. Both alleles of a gene can be inactivated with high frequency with these nucleases. Especially with CRISPR/Cas9, mammalian genome engineering has become simple, reliable and cheap [19].

Glutamine synthetase knockout cells

The glutamine synthetase (GS) gene is used widely as a selection marker for stable CHO cells. Cells overexpressing GS can be selected by inhibiting the endogenous glutamine synthetase with the inhibitor methionine sulfoximine (MSX). The GS marker and MSX-selection of stable CHO cell lines were used to produce protein for structure determination of the ICAM-5 ectodomain [20], a TCR:MHC:antigen heterotetramer [21], aminopeptidase N [22], the insulin receptor ectodomain [23] and acetylcholinesterase [24]. A novel CHO host cell was created by knocking out the GS gene and the GnTI gene with specific zinc finger nucleases [25•]. With GS-deficient CHO host cells, MSX selection of cells stably transfected with a GS vector is much more efficient than with normal cells. The selection of low-producing cell clones was largely prevented by using a GS-deficient host cell line [26]. Moreover, among MSX-selected, stably transfected cell clones, a six-fold higher proportion of top producing clones (>2 g/L) was found when a GS-deficient host cell line was used, in comparison to the original host cell line.

New cell lines developed by sequence-specific genome engineering such as GnTI and GS double knockouts are potentially useful for structural biology. Genome engineering could also be used for removing genes of

unwanted host cell proteins that are co-purified with the target protein.

Methods of stable cell line generation

GFP and cell sorting

Transgene insertion into a host cell chromosome upon transfection is a rare event and, to make things worse, most of the integrated transgenes will be inactivated by epigenetic mechanisms. Cells that received an active genome-integrated transgene therefore have to be selected by a marker. Antibiotic resistance is commonly used, but can be an unreliable reporter for high level, uniform transgene expression [27]. Green fluorescent protein (GFP) is a useful alternative [28]. Cells that stably integrate a GFP expression vector are directly identified by intracellular fluorescence and can be isolated by preparative FACS cell sorting. Cell sorting can isolate a small number of high-producer clones from millions of cells. By repeating the process at different time points, cells that express GFP stably over time can be isolated. By this method, clonal cell lines with constantly high GFP expression over several months can be obtained without applying selective pressure [27].

GFP expression can be coupled to expression of the gene of interest by constructing internal ribosome entry site (IRES)-based bicistronic vectors comprising the gene of interest and a GFP gene. IRES-based GFP co-expression in combination with cell sorting and antibiotic selection was used to generate a stable HEK293S GnTI$^-$ cell line for production of a soluble integrin $\alpha_X\beta_2$ heterodimer for structure determination [29]. In an alternative approach, the GFP gene can be excised by site-specific recombination upon clone isolation, thereby bringing a gene of interest, located downstream, under the control of the promoter driving transgene expression (Figure 2) [27,30].

Cell sorting may require optimization to maintain cell viability. In our own experience, the viability of CHO Lec3.2.8.1 cells grown in suspension can be low upon cell sorting. Shear stress was reported to cause low cell viability upon sorting of insect cell lines. Addition of Pluronic acid F-68 improved the survival of sorted insect cells [31•] and may also have a positive effect on mammalian cells.

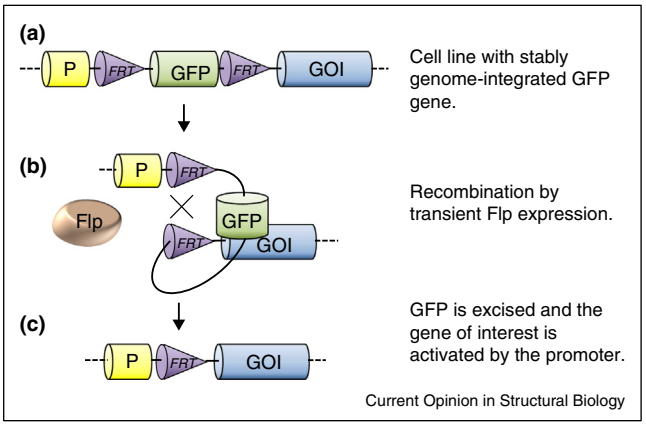
Secreted GFP fusion proteins

A secreted GFP marker was used for studying the structure of a sialyltransferase [32•]. The sialyltransferase was fused to a codon-optimized, folding-enhanced GFP version called 'superfolder' [33]. The fusion protein was secreted by a stable HEK293S GnTI$^-$ cell line with high yield (75 mg/L). The GFP tag allowed for direct protein quantification by fluorescence spectroscopy during cell line development and protein purification. It was removed by proteolysis from the sialyltransferase before crystallization.

Recombinase-mediated cassette exchange

The productivity of a stable cell line depends on the genetic locus of transgene integration. Instead of random integration, it would be desirable to target the transgene to a specific locus that allows for strong and stable transgene transcription. This can be achieved by recombinase-mediated cassette exchange (RMCE) using site-specific recombinases [34,35]. RMCE requires a 'master' cell line carrying a single copy of a reporter gene at a suitable genetic locus. By RMCE, the reporter gene is exchanged against the gene of interest (GOI). For RMCE with the

Figure 2



Replacing a GFP marker with a gene of interest by Flp-mediated excision for generating stable producer cell lines [27]. **(a)** First, a stable GFP-positive cell line is established by cell sorting. **(b)** Then, the GFP gene is replaced by the gene of interest. This is achieved by transient overexpression of Flp recombinase. Flp mediates recombination of the *FRT*-sites flanking the GFP-gene, which leads to the gene's excision. **(c)** The gene of interest now becomes activated by the transgene's promoter. P: promoter, GOI: gene of interest, *FRT*: Flp recognition target site.
Figure adapted from Wilke *et al.* [30].

site-specific Flp recombinase, the reporter gene is flanked by two distinct Flp recognition target (*FRT*) sites that have been engineered so that they cannot recombine with each other (Figures 3 and 4). RMCE is initiated by introducing the Flp recombinase and a vector with the gene of interest (GOI), flanked by *FRT* sites, into the master cell line. Recombination of *FRT* sites leads to exchange of the reporter gene and the GOI, placing the GOI at a highly active, stable genetic locus (Figure 4). The same master cell line can be used for generating producer cell lines for many different target proteins [34,35].

The stable CHO Lec3.2.8.1 cell line SWI3a-26 is a master cell line for RMCE that was generated by random integration of a *FRT*-flanked GFP reporter gene [36]. It contains a single copy of the GFP reporter transgene at a genetic locus that is protected from silencing. The integrated GFP cassette contains a 'selection trap' that allows for selection of recombinant cells upon RMCE [37]. The selection trap is an inactive selection marker, lacking a promoter and a start codon (Figure 4). It is complemented upon RMCE and allows for antibiotic selection of recombinant cells. Using SWI3a-26, production cell lines were established by RMCE for different mammalian glycoproteins [36,38], including the ectodomain of the lysosomal membrane protein DC-LAMP. In consequence, the DC-LAMP domain structure was solved by X-ray crystallography [39•].

In our experience, RMCE takes about 7 weeks from the day of transfection to cryopreservation of clonal production cell lines [36]. In comparison to random integration, the process is faster and the effort of screening of large numbers of clones is avoided. The multi-host expression vector pFlpBtM allows for protein production in *E. coli*, transiently transfected mammalian cells and Baculovirus-infected insect cells and for construction of stable cell lines by RMCE with a single vector [38].

RMCE-derived production cell lines contain only a single copy of the transgene. Nevertheless, high-yield antibody production of up to 2 g/L shake flask culture was achieved with an RMCE system that uses Cre-*lox* recombination [40•]. With this system, about 5 weeks were required from transfection to completion of stable pool production cultures.

Two independent genetic loci can be targeted with two different transgenes by RMCE with a single transfection. This has been achieved by designing new synthetic *FRT* site variants [41].

Transfection efficiency and stable pools

Transfection of HEK293 and CHO cells leads to integration of transgene DNA at random chromosomal loci, but the frequency of these integration events is very low with commonly used vectors. Highly efficient systems for chromosomal integration of transgenes accelerate cell line generation and allow for protein production with stable pools, thereby eliminating time-consuming cloning steps. For protein production with a stable pool, the bulk of stably transfected cells are selected and used directly for protein production. The structure of lysosomal integral membrane protein (LIMP2) was solved using a stable pool obtained by transfection with the ΦC31 integrase system (described below) and antibiotic selection [42]. Efficient chromosomal integration is also achieved with Lentivirus particles and transposons (Figure 3).

Lentivirus

Lentiviral transduction of mammalian cells is very efficient and highly productive cells are generated at a high frequency. The usefulness of the Lentivirus system was demonstrated by establishing stable cell lines and stable pools for production of antibodies and blood coagulation factor VIII [43,44,45]. The Lentivirus efficiently transports the transgene cDNA into the nucleus, where it is integrated into the host cell genome by the viral integrase (Figure 3). A stable cell line for production of an IgG receptor subunit, which resulted in crystal structure determination, was established with a recombinant Lentivirus [46]. Stable cell line generation by Lentivirus transduction and by non-viral plasmid transfection was compared [45]. Gene delivery into nearly 100% of CHO cells grown in serum-free suspension culture was obtained by Lentivirus. GFP overexpression was up to five times higher in comparison to plasmid transfection. Potential drawbacks of the Lentivirus system are safety concerns, the error-prone replication of the viral RNA genome by reverse transcription and the extra step of virus particle preparation.

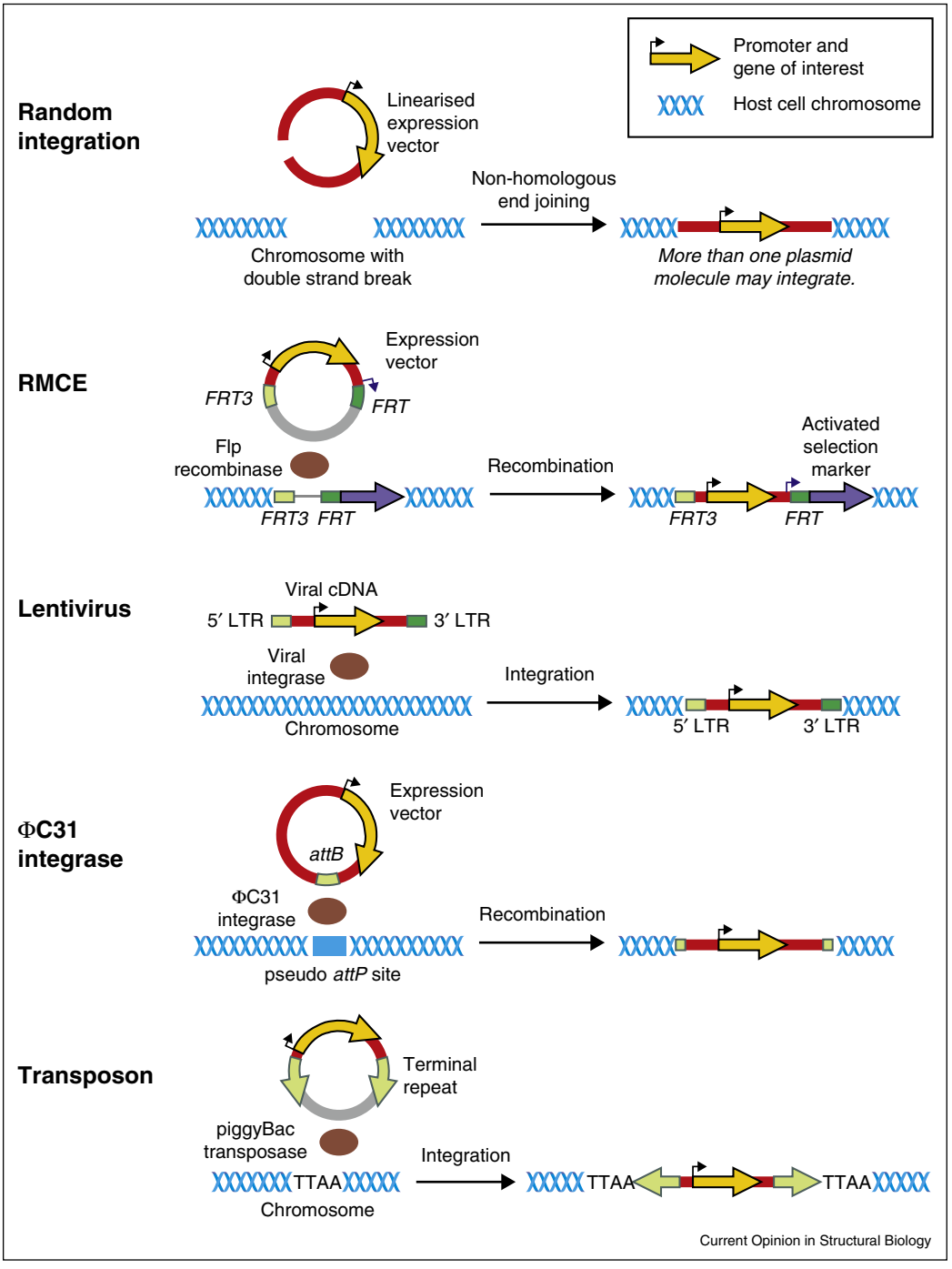
Phage ΦC31 integrase

The integration system of the *Streptomyces* phage ΦC31 represents a non-viral alternative for active transgene integration. The ΦC31 integrase performs recombination between the *attP* site of the phage genome and the *attB* site in the host bacterial chromosome. In mammalian cells, it mediates integration of plasmids bearing an *attB* site into chromosomal sequences that have sequence similarity with *attP*, termed pseudo *attP* sites [47]. Stable mammalian cell lines are generated by co-transfection with a ΦC31 integrase expression vector and an expression vector for the gene of interest that has an *attB* site (Figure 3). This system was used for protein production and structure determination of LIMP-2 [42], tumor antigen 5T4 [48] and acetylcholinesterase [49].

Transposons

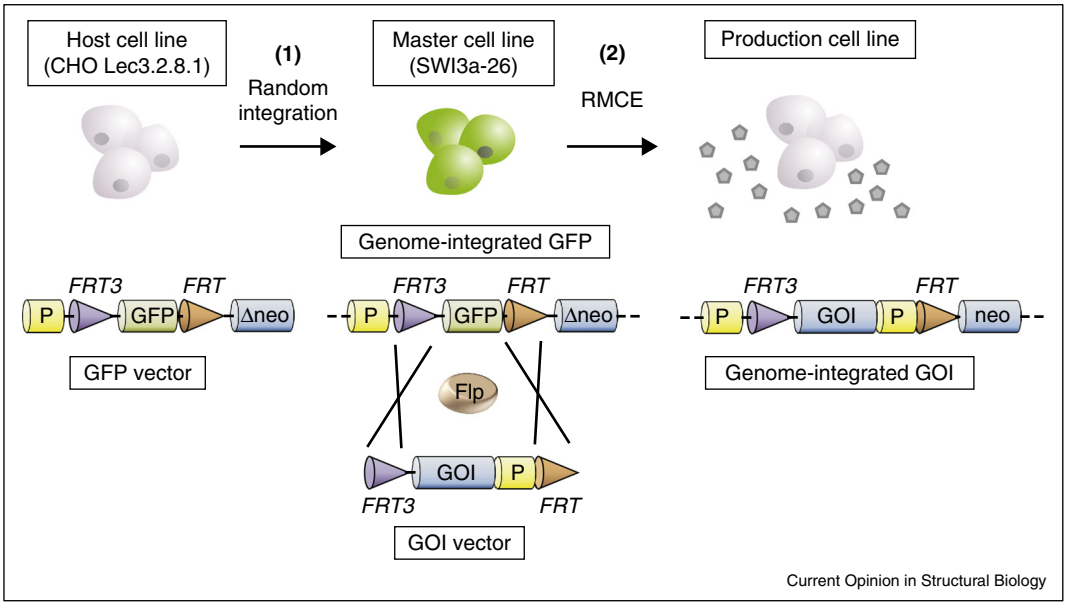
High rates of chromosomal integration have also been achieved with transposon vectors. The terminal inverted repeats of the 'piggyBac' transposon are recognized by the transposon's integrase, which leads to integration of the flanked sequence into a chromosomal TTAA site

Figure 3



Techniques of transgene genome integration for generating stable cell lines. *Random integration*: The linearized expression vector with the gene of interest (yellow) is integrated by non-homologous end joining into the host cell genome at the position of a random chromosome break. *RMCE* (recombinase-mediated cassette exchange): A master cell line, engineered to carry *FRT* sites at a suitable locus of its genome, is co-transfected with a vector with an *FRT*-flanked gene of interest (GOI) and a second vector for overexpression of Flp recombinase. The Flp recombinase catalyses site-specific genome integration of the GOI (see also Figure 4). *Lentivirus*: The host cell line is transduced by a Lentivirus that carries the GOI in its genome. The viral RNA genome is reverse-transcribed into cDNA, which is integrated into the host cell genome by the viral integrase at random locations. *ΦC31 integrase*: The host cell line is co-transfected with an expression vector for phage ΦC31 integrase and an expression vector for the GOI which contains an *attB* recombination site. The ΦC31 integrase is expressed and catalyses recombination of the *attB* site with genomic sequences that resemble the *attP* recombination site (pseudo *attP* sites). *Transposase*: The host cell line is co-transfected with an expression vector for the transposase of the piggyBac transposon and a vector containing the GOI, flanked by the transposon's inverted terminal repeats. The integrase recognizes the repeats and integrates the flanked sequence into a chromosomal TTAA site.

Figure 4



Recombinase-mediated cassette exchange (RMCE) with the CHO Lec3.2.8.1 cell line SWI3a-26 [36]. **(1)** The SWI3a-26 master cell line was generated from CHO Lec3.2.8.1 cells by random integration of a vector with a GFP reporter gene, flanked by a wild-type *FRT* site and a synthetic variant (*FRT3*). The cell line was isolated by cell sorting and contains a single copy of the GFP vector integrated at a genomic locus that allows for high-level gene expression. The GFP vector contains a selection trap consisting of an inactive neomycin resistance gene, Δneo, that lacks a promoter and a start codon. **(2)** Cassette exchange is initiated by co-transfecting the master cell line with vectors for the Flp recombinase and the gene of interest (GOI), flanked by compatible *FRT* sites. The transiently expressed Flp recombinase exchanges the *FRT*-flanked cassettes. The selection trap is triggered by activating the Δneo gene with a promoter and a start codon from the GOI vector, thereby enabling selection of recombinant clones.
Figure adapted from Wilke *et al.* [36].

(Figure 3). In stable CHO cell development, the piggyBac system strongly increased the frequency of stable integration and lead to up to fourfold higher protein yield from pools of transfected cells [50]. Similarly positive results were obtained with the 'Sleeping Beauty' transposon system and HEK293 cells [51]. A stable cell line overexpressing the four subunits of a γ-secretase complex was established in one step with the piggyBac system [52••]. A newly designed vector set for doxycycline-inducible overexpression utilizes multiple-copy integration by the piggyBac integrase and was used with HEK293S GnTI$^-$ cells for high-level secretion of 14 proteins with stable pools [53•].

Conclusions

Protein production with stable cell lines for structural biology relies strongly on glycosylation-deficient host cells. A novel HEK293 cell line, called 'GlycoDelete', was equipped with an intracellular endoglycosidase for secretion of deglycosylated glycoproteins. Host cells can be improved by sequence-specific genome engineering, allowing for efficient and highly specific knock out of multiple genes. A CHO cell was reported that lacks both GnTI and glutamine synthetase (GS) activity, which allows for highly efficient selection of stable cell lines with GS markers.

A survey of recent reports of protein crystal structures indicated that transient transfection of mammalian cells and stable mammalian cell lines were used with around the same frequency. In comparison to transient transfection protocols, the generation of stable cell lines has several bottlenecks. The frequency of stable genome integration upon transfection with plasmid vectors is low and, moreover, most integrated transgenes will be silenced. Top producers are usually rare in the pool of stably transfected cells and their identification requires isolating and characterizing a large number of clones.

Techniques for improved genome integration of transgenes and for improved selection of high-producing stable cell lines have been developed. Using fluorescent proteins as selection markers allows for isolating high-producing stable cells among millions of transfected cells by cell sorting. Novel glutamine synthetase knock out cells generated by sequence-specific genome engineering allow for a more efficient selection of high-producer cells with the glutamine synthetase selection marker. The problem of transgene silencing has been addressed by the recombinase-mediated cassette exchange (RMCE) technique. Here, site-specific recombination is used for targeting the gene of interest to specific genetic loci that

are protected from silencing and that allow for high-level gene expression.

Plasmid DNA introduced into the nucleus of host cells is integrated into the genome by host cell factors at random sites with a low frequency. Much more efficient genome integration is achieved when additional, heterologous integration factors are introduced. This is accomplished with Lentiviruses, piggyBag and Sleeping Beauty transposons and the phage ΦC31 integrase. Transfection of host cells with these highly efficient systems results in a pool of cells with a high proportion of stable high-producers. Such a stable pool can be used directly for protein production, thereby avoiding time-consuming clone isolation and characterization steps.

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- • of special interest
- •• of outstanding interest

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