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Stability of single copy transgene expression in CHOK1 cells is affected by histone modifications but not by DNA methylation

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ABSTRACT

Intraclonal heterogeneity of genetically modified mammalian cells has been observed as a phenomenon that has a strong impact on overall transgene expression levels and that limits the predictability of transgene expression in genetically modified cells, thereby hampering single cell based screening approaches. The underlying mechanism(s) leading to this variance are poorly understood. To study the dynamics and mechanisms of heterogeneity of early stage silencing we analyzed the expression in more than 100 independent clones of CHOK1 cells that harbour genetically stable integrates of single copy reporter cassettes driven by EF1α and CMV promoters. Single cell analysis showed intraclonal variability with heterogeneity in expression in genetically uniform populations. DNA methylation is a well known mechanism responsible for silencing of gene expression. Interestingly, loss of expression was not associated with DNA methylation of the CMV promoter. However, in most of the clonal populations expression could be increased by inhibitors of the histone deacetylases (HDACi) suggesting that heterogeneity of transgene expression is crucially governed by histone modifications. Further, to determine if the epigenetic status of transgene expression is governed by the chromosomal integration locus we targeted heterologous expression cassettes into two chromosomal sites using recombinase mediated cassette exchange (RMCE). The expression status of a particular clone was faithfully re-established when the same promoter used. In this way the problem of early stage cell clone instability can be bypassed. However, upon introduction of an unrelated promoter methylation-independent silencing was observed. Together, these results suggest that histone modifications are the relevant mechanisms by which epigenetic modulation of transgene expression cassettes is governed in the early phase of clone generation.

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1. Introduction

Transcription is a major level at which mammalian gene expression is governed. The transcriptional activity of genes is controlled not only by the proximal regulatory promoter elements but also by remote regulatory DNA regions. A number of chromosomal elements have been identified that influence the activity of individual promoters. These include enhancers, silencers, insulators (Phillips-Cremins and Corces, 2013; West and Fraser, 2005; West et al., 2002), scaffold/matrix associated regions (S/MARs) (Bode et al., 2000; Galbete et al., 2009), locus control regions (LCRs) (Koh et al., 2010) and ubiquitously acting chromatin opening elements (UCOEs) (Zhang et al., 2010) (for a recent review comprising all these elements see Kruse et al., 2014). Some of these elements are thought to interact via looping of the DNA within a chromosome and also interchromosomal interactions have been suggested (Williams et al., 2010). The delicate regulation via proximal and remote sequences of individual genes is highly organized and is considered to be the result of the evolutionary process.

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Most protocols for transgene introduction into cells rely on random DNA integration, frequently also resulting in unpredictable numbers of integrated transgene copies at different sites. Once integrated, control and stability of transgene expression is not only affected by the proximal regulatory elements of the incoming expression cassettes but also by the remote chromosomal elements that flank the transgene. The strength of transgenic promoters is affected by various parameters. First, it depends on the cell-type specific set of available transcription factors that bind to the regulatory elements. Second, it is strongly influenced by the genetic setting governed by the chromosomal integration site. Finally, the strength of promoters depends on the accessibility of the DNA, i.e. its chromatin state (Schubeler, 2012; Tiwari et al., 2008) which is critically affected by epigenetic modifications. Such modifications include the methylation pattern of DNA as well as specific histone modifications such as methylation and acetylation on the specific amino acid residues (Ghavifekr Fakhr et al., 2013). Thereby, differential accessibility and/or binding of DNA sequences by the set of different factors can occur. Together, this modulates the efficiency of gene transcription (Buck et al., 2013; Cui et al., 2013).

DNA methylation is one of the best characterized epigenetic modifications. It predominately involves addition of a methyl group to the position 5 of cytosine residues that are coupled to guanine (CpG motifs) (Crider et al., 2012). Histone modifications comprise a set of modifications like methylation and acetylation that can occur on specific residues of amino acids (e.g. lysines) present on the histone tails. These modifications form a histone code that modulates gene expression by allowing or preventing access to chromatin, thereby acting as a guide for the transcription factors and other regulatory proteins. Recently, a number of studies have permitted a partial unravelling of this code (Gacek and Strauss, 2012; Long et al., 2004; Misri et al., 2008). Generally, DNA hypermethylation and certain histone markings like trimethylation of lysine 27 on histone H3 have been considered to suppress gene expression, whereas the DNA hypomethylation and histone H3 acetylation was shown to be associated with active gene expression (Connolly et al., 2013; Kirmizis et al., 2004; Pauler et al., 2009; Yu et al., 2007).

Most of our understanding of the epigenetic role in differential gene expression is based on studies of cellular genes in their natural environment. In contrast, much less is known about the crosstalk of heterologous transgenic regulatory elements upon integration into a specific chromosomal domain as it occurs upon stable genetic modification of cells, e.g., during the establishment of production cell lines. Indeed, once transgenes are integrated into the chromosomal DNA, a high clone-to-clone variation of transgene expression is usually observed. This is generally described as the chromosomal 'position effect' (Batenchuk et al., 2011; Chen et al., 2013; Daboussi et al., 2012; Dag et al., 2014; Yin et al., 2012). The ill-defined position effect describes the influence of all proximal and remote cis-acting chromosomal elements which modulate binding of transcription factors and thereby also modulate the strength of the introduced transgene cassette.

A major challenge in the establishment of transgenic cells and animals is the fact that transgenes are frequently silenced upon stable integration into the host's chromosomes (Duan et al., 2012; Kong et al., 2011; Kues et al., 2006). This also applies to CHOK1 cells, a cell line used for protein production (Osterlehner et al., 2011). Various mechanisms have been identified that contribute to the loss of transgene expression. In CHO cell clones in which transgene expression was established upon gene amplification expression instability is associated by chromosomal rearrangements and recombinations, leading to reduction or even complete loss transgene copy numbers (Du et al., 2013; Fann et al., 2000; Kim et al., 1998a, 1998b) reviewed in Barnes et al. (2003). Single copy genes are less affected by this type of karyotypic instability. Also DNA methylation of the transgene was found to be associated with loss of expression, particularly in multi-copy integrations (Hsieh and Fire, 2000; McBurney et al., 2002; Mielke et al., 2000; Osterlehner et al., 2011; Yang et al., 2010). Other mechanism that has been reported to compromise the expression include the apoptotic stress on the cell leading to loss of expression (Goswami et al., 1999; Kim et al., 2013).

Most studies investigating silencing in CHO cells focus on the late phases of clone generation that happens, e.g., during establishment of cell banks. Usually, these studies rely on cell clones that were established upon stringent selection regimens – a standard procedure in clone generation. However, selection regimens can bias the investigation of silencing. Moreover, selection protocols also enrich cell clones in which the chromosomal status does not per se provide optimal conditions but rather require continuous pressure to permanently select for cells in which the chromosomal integration site is in a transcriptionally active status. Also, in most of these studies the copy-number was not controlled and as a consequence silencing mediated by integration of cassette repeats cannot be excluded. Thus, although the standard methods for clone generation proved to be beneficial for establishing high titre clones, they seem to be less favourable to study general mechanisms of silencing as a consequence of crosstalk between the incoming cassette and the chromosomal environment.

We wanted to elucidate the early cues leading to transgene silencing and clonal heterogeneity in the absence of selection pressure. To elucidate these mechanism(s) we investigated more than 100 clones with single copy transgene cassettes in random chromosomal sites of CHOK1 cells. We identified high interclonal but also intraclonal heterogeneity. Contrary to our expectations that these alterations in expression along with complete loss in expression might be modulated by DNA methylation, we found histone modifications to be responsible for the heterogeneity. While the epigenetic pattern was found to undergo dynamic alterations during cultivation expression could be re-established upon targeted cassette exchange.

2. Materials and methods

2.1. Mammalian cell culture and vectors

CHOK1cells (ATCC CCL61) were cultivated at 37 °C in a humidified atmosphere with 5% CO_2. To culture CHOK1 cell line we used DMEM medium and Ham's-F12 medium (Invitrogen) in a ratio of 1:1. Culture media were supplemented with 10% foetal calf serum, 2 mM L-glutamine, Penicillin (10 U/ml) and streptomycin sulfate (100 μg/ml).

The lentiviral self-inactivating tagging vectors (pTAG CMV GFP and pTAG EF1a GFP) are based on pJSARGFP (May et al., 2008). In these vectors, the human CMV and the human EF1a promoter, respectively, drives GFP. The lentiviral SIN backbone of pJSARGFP (ClaI-NheI fragment) comprises the LTRs, REV responsive element and Woodchuck hepatitis virus regulatory element (WPRE) was ligated to a reporter cassette encoding the eGFP reporter gene controlled by the CMV promoter. This cassette was further flanked by a set of heterospecific, non-interacting Flp recombination-target sites FRT-WT and FRT-F5 (Fig. 1A).

The targeting vectors pTAR CMV RFP and pTAR SV40 RFP were derived from pEMTAR (Schucht et al., 2006). Briefly, cassettes comprising a CMV-RFP and SV40-RFP expression cassette were integrated into the NaeI site of pEMTAR, thereby flanking the expression cassette with FRT-WT and FRT-F5 mutant sites.

Maps and vector sequences are available on request.

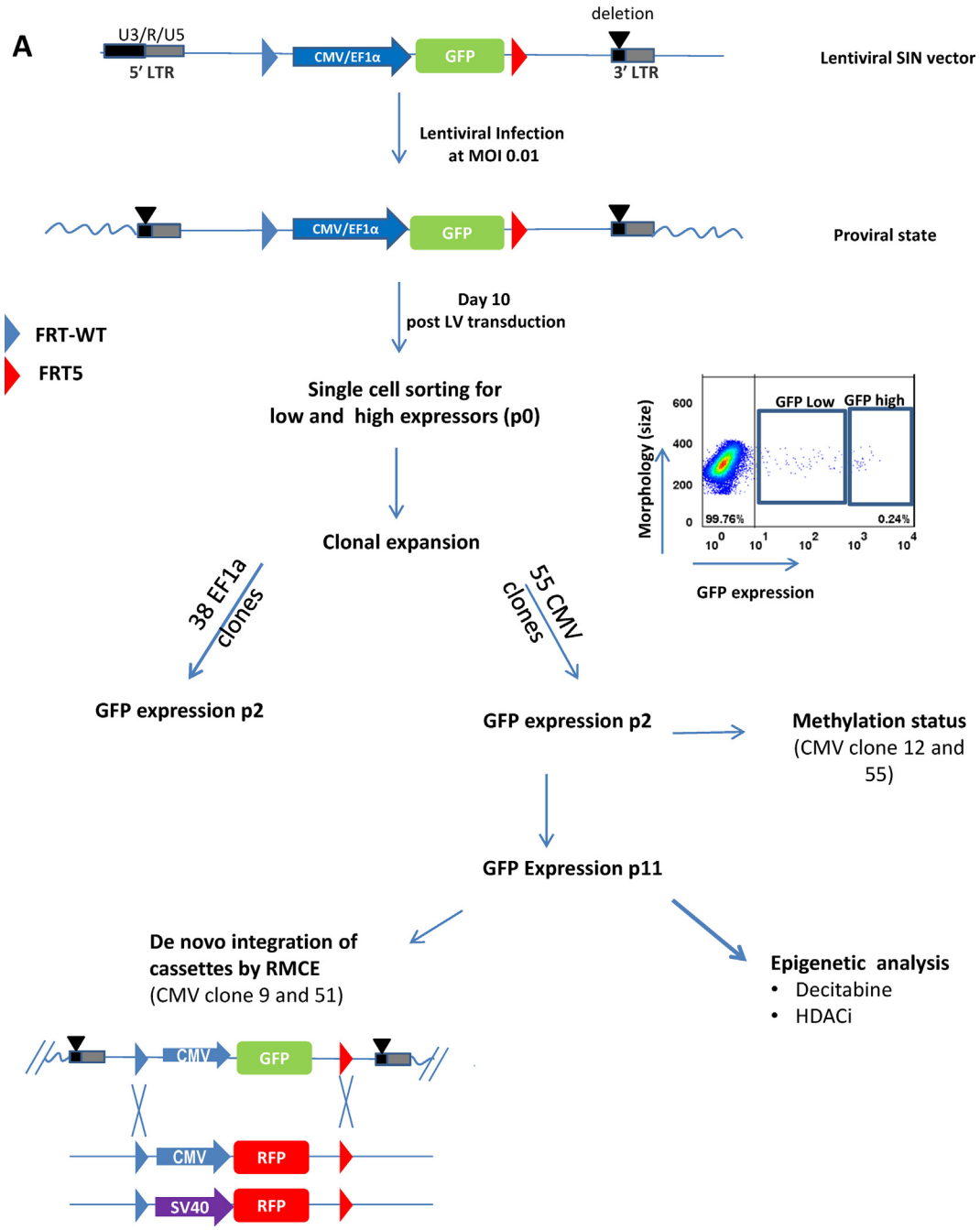


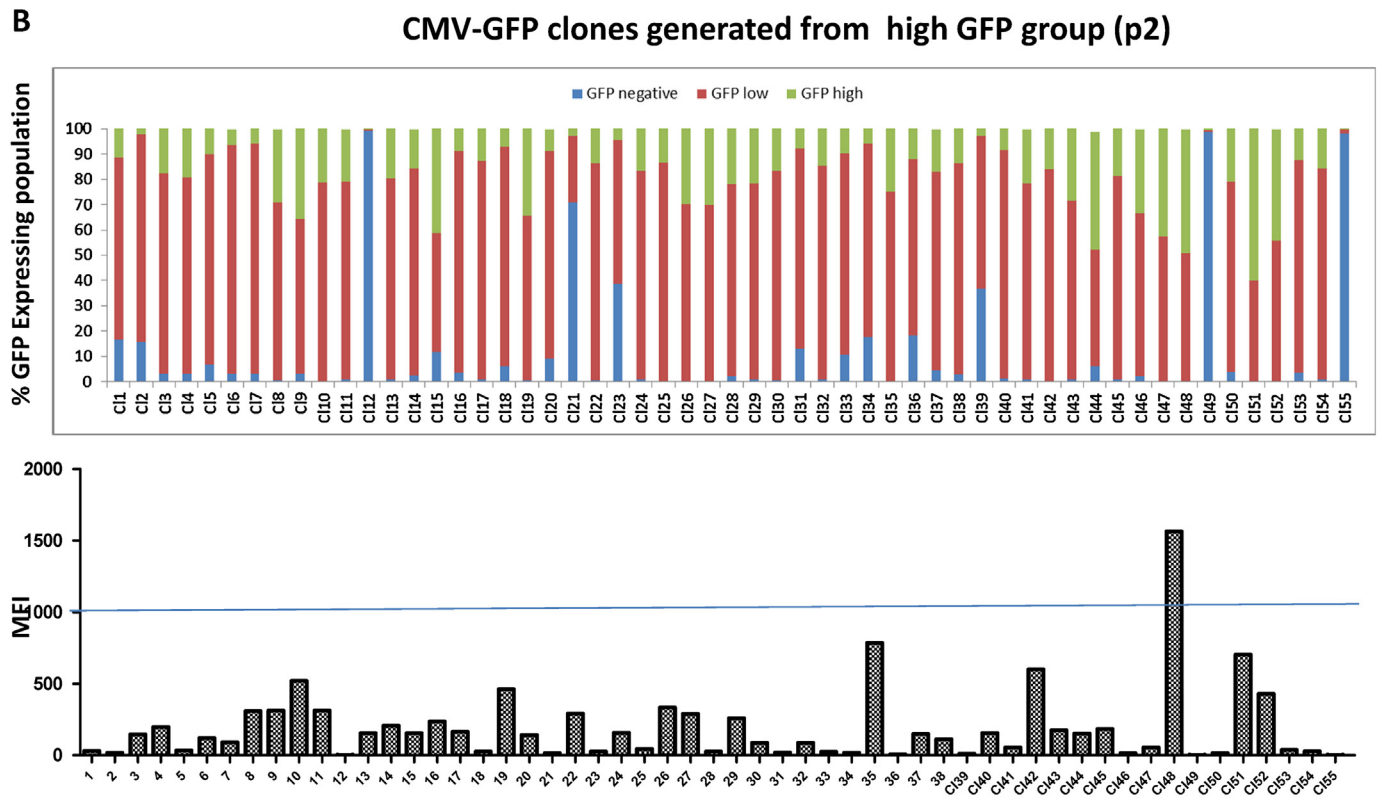
Fig. 1. Generation of single copy cell clones and expression level at p2. (A) Overall strategy followed in this study (see text for details). (B–E) GFP expression level and overall mean fluorescence intensity (MFI) of isolated clones at p2. Independent clones derived from CHOK1 CMV-GFP (B, C) and EF1α-GFP (D, E) infected cells upon sorting for high ($>10^3$ a.u.) and low (10^1–10^3 a.u.) expressing cells ('high' (B, D) and 'low' (C, E) group, respectively). Clonal fractions with no detectible GFP expression ($<10^1$) are depicted by red, low (10^1–10^3) are shown by red and high ($>10^3$ a.u.) are indicated in blue.

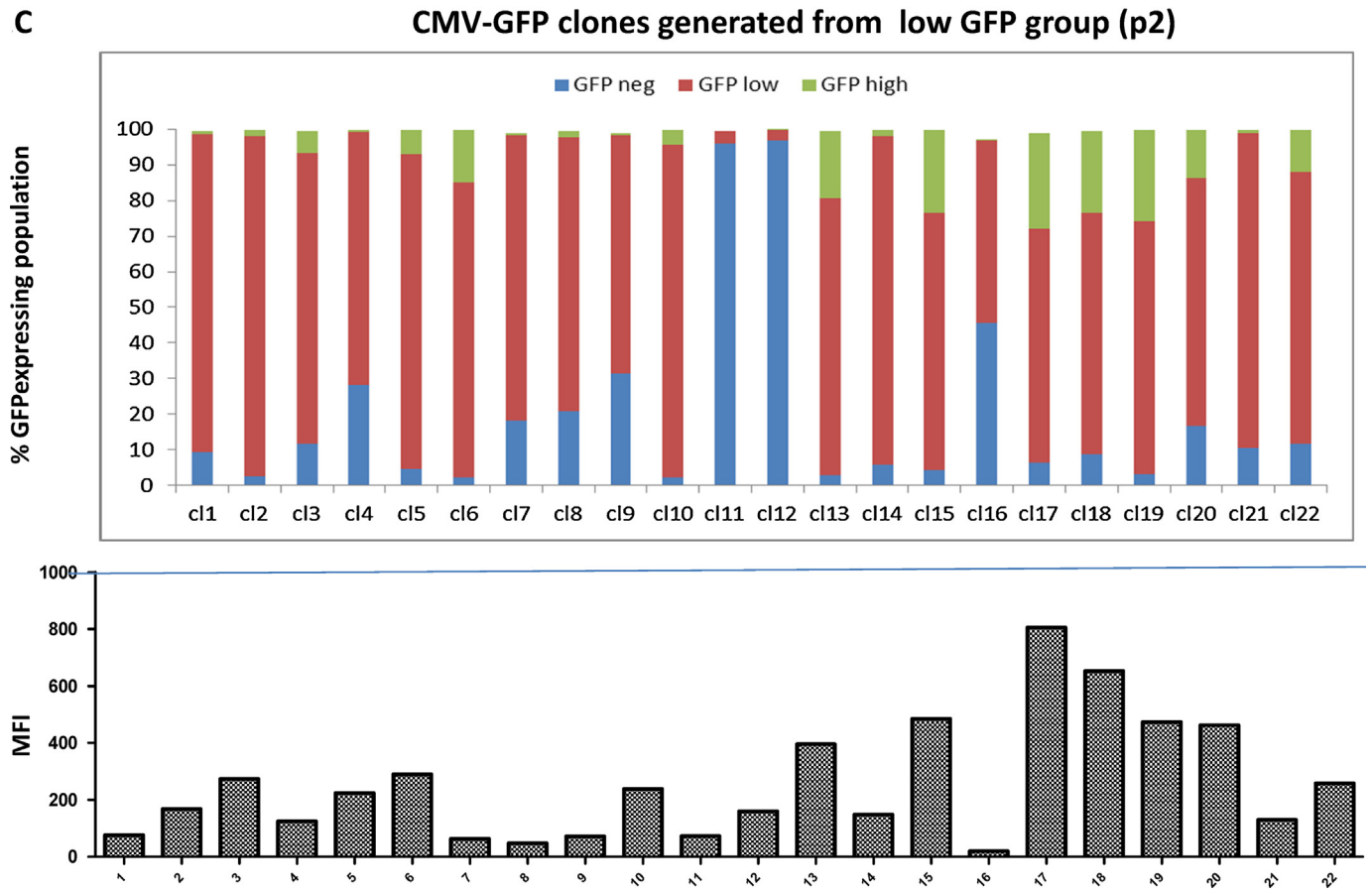
2.2. Lentiviral gene transfer

HEK293T cells were used for lentivirus production as specified in Gama-Norton et al. (2011). Briefly, HEK293T cells were transfected using the calcium phosphate method with four different plasmid constructs: the tagging vector (pTAG CMV GFP), envelope-encoding plasmid (pLP-VSVG), gag/pol helper plasmid (pLP1) and REV expressing plasmid (pLP2) (all helper plasmids were obtained from Invitrogen). After 12 h the medium was changed. 48 h after transfection the supernatant containing the lentiviral particles was harvested and filtered through a 45 μm filter. The virus supernatant was titered by infecting 293T cells with serial dilutions. For generation of single copy tagged clones, 50.000 CHOK1 cells per 12 well were seeded 24 h before lentiviral transduction and infected with 1000 viruses (MOI 0.01) in the presence of 8 μg/ml protamine sulphate (Sigma–Aldrich). The day after infection, medium was exchanged.

2.3. Recombinase mediated cassette exchange (RMCE)

Targeting of the tagged clones was done by Flp mediated recombinase cassette exchange according to previously published





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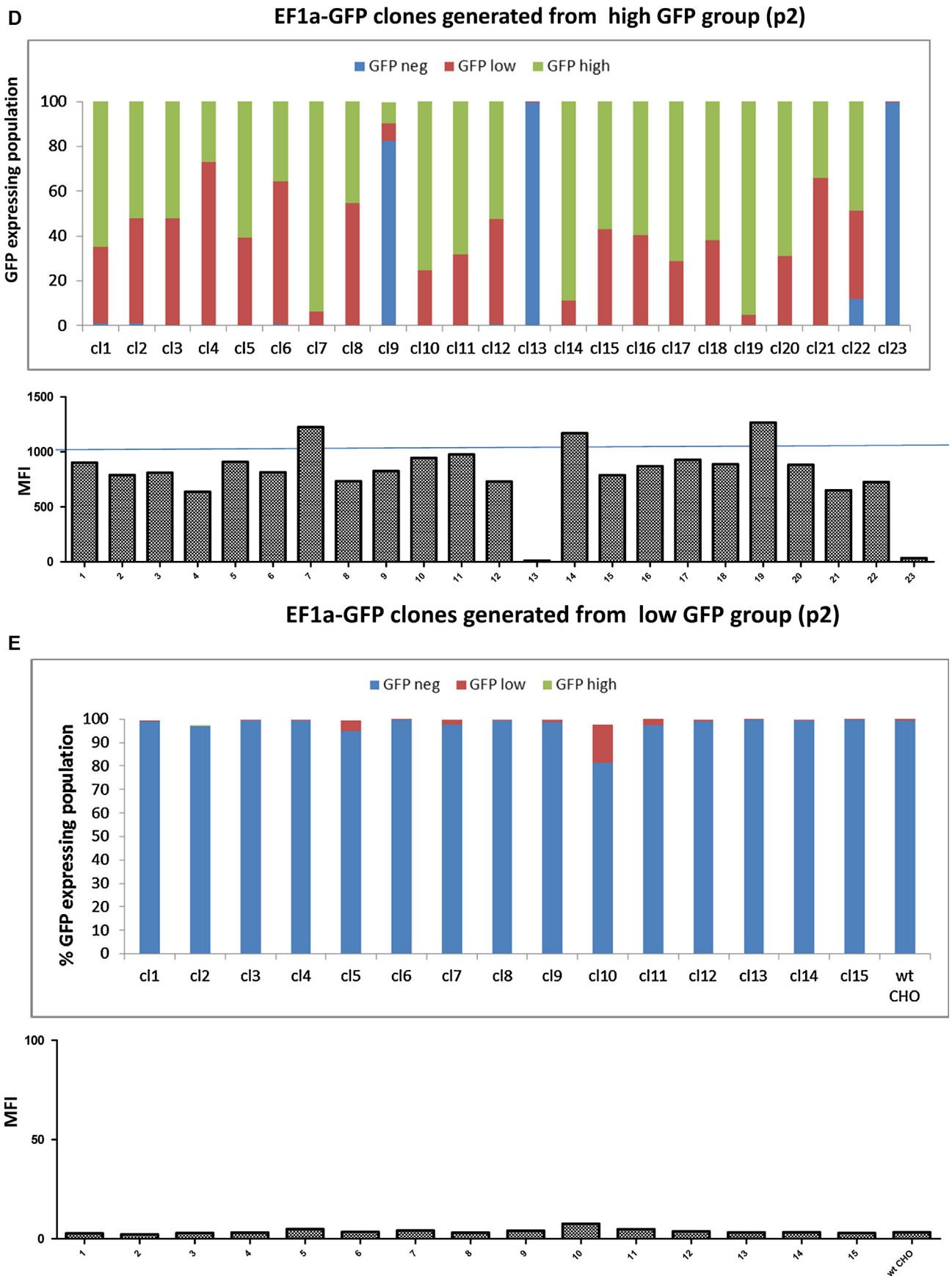


Fig. 1. (*Continued*)

protocols (Nehlsen et al., 2009). Briefly, the FLPe expression vector pFlpe (0.7 μg) (Nehlsen et al., 2009) was cotransfected with the targeting vectors pTAR CMV RFP (2.1 μg) and pTAR SV40 RFP (2.1 μg), respectively, using lipofectamine 2000 (Life Technologies) according to manufacturer's protocol. Media was exchanged after 5 h. The cells were then cultured for 10 days after transfection and then were sorted for lack of GFP expression resulting from successful RMCE. Targeted vector integration was confirmed by PCR.

2.4. Chemical treatments

For the chemical treatments 1×10^5 cells were seeded in a 24-well plate format. HDAC inhibitors sodium butyrate (Sigma–Aldrich) and Valproic acid (Sigma–Aldrich) were dissolved in PBS and added at a final molecular concentration of 1 μM and 300 mM, respectively. Treated and untreated control cells were cultivated for 72 h and then harvested for further analysis.

For treatment with the Dnmt inhibitor (Dnmti) Decitabine, 1×10^5 cells were seeded in a 12-well plate format. Decitabine (Sigma–Aldrich) was dissolved in PBS and added at a final molecular concentration of 1 μM. Treated and untreated control cells were cultivated for 72 h and then harvested for further analysis.

2.5. Flow cytometry

Flow cytometry was used for the analysis of transgene expression with BD FACScalibur. Sorting of the cells was done with FACSaria, using the 488 nm laser and 525/50 nm filter setting for GFP and the 561 nm laser and 585/15 nm filter for RFP. A gating strategy was used to eliminate doublets and dead cells or debris. Results were quantified with the FlowJo 7.6 software.

2.6. Bisulfite sequencing

All reagents were obtained from the EZ DNA methylation kit (Zymo). The protocol was followed according to the manufacturer's instructions. Bisulfite converted DNA was amplified with bisulfite primers specific for CMV promoter (forward primer 5′-GTATATGATTTTATGGGATTTTTTTATTTG-3′ and reverse primer 5′-ATTCACTAAACCAACTCTACTTATATAAAC-3′) and for SV40 promoter (forward primer 5′-AAGGGAATAAAAGTTGGAGTTTTAT-3′ and reverse primer 5′-TCACTACTTCTAAAATAACTCAAAAACC-3′). The reaction was performed on PCR machine (Biometra) according to the following conditions: (1) Pre incubation: 95 °C 15 min. (2) Amplification: (a) Denaturation 95 °C for 30 s. (b) Annealing 55 °C 60 s. (c) Elongation 72 °C 60 s (45 cycles). (3) Final elongation 72 °C for 7 min. (4) Final hold 4 °C. Amplified PCR products were integrated into the PCR blunt cloning vector (Invitrogen) using the protocol according to the manufacturer's instructions. Upon transformation, single independent clones were picked and expanded. Plasmid DNA was isolated, purified and sequenced. Sequences were then analyzed for the presence of C to T conversions indicating unmethylated CpGs.

3. Results

3.1. Sorting strategy and transgene expression from EF1a and CMV promoter

To study expression from defined chromosomal loci in CHOK1 cells, we generated cell clones with random integration of a single copy expression cassette in the absence of selection pressure. Both, the human cytomegalovirus (CMV) promoter and the human EF1α promoter were used to drive the expression of GFP as a sensitive cell based reporter. As a reliable method for achieving single copy integrations lentiviral transduction was used. A self-inactivating (SIN) lentiviral vector with a deletion of the viral promoter in the 3′LTR was employed which avoids interference of the viral regulatory elements with the promoter upon infection (Fig. 1A). In addition, the reporter cassette was flanked with a set of heterologous FRT sites (FRT-WT and FRT-F5) which provides the option for subsequent exchange of cassettes by Flp recombinase mediated cassette exchange (Turan et al., 2013; Wirth et al., 2007). To ensure single copy integration of the screening cassette, infection was performed at a multiplicity of infection (MOI) of 0.01. Thereby, statistically, 99% of infected cells carry a single copy integration of the expression cassette. 10 days after lentiviral infection (defined as p0), cells showing either 'low' GFP expression (10^1–10^3 arbitrary units, a.u.) or 'high' GFP expression (>10^3 a.u.) were isolated by single cell sorting (Fig. 1A). Individual cell clones were established from single cells and expanded for further analysis.

125 such cell clones from CHOK1 cells were randomly selected for further characterization. We analyzed the GFP expression immediately after sorting (p2) by flow cytometry analysis (Fig. 1B–E). To reach this state, 17 cell generations were required. For the EF1α driven cassettes, 87% (21/23) of clones established from the high GFP expressing cells displayed GFP expression but almost all (14/15) clones that were established from the low GFP expressing cells showed less than 10% GFP expressing cells. On the contrary, the CMV driven transgene expression was much more stable during the establishment of clones with 91% (20/22) and 95% (52/55) GFP expressing clones generated from low and high expressing cells, respectively (Fig. 2A).

We categorized the cell clones at p2 with respect to the mean fluorescence intensity (MFI) of the overall population as well as the percentage of cells providing high (>10^3 a.u.), low (10^3–10^1 a.u.) and undetectable GFP expression (<10^1 a.u.). Strikingly, from the fraction of 'high' expressing cells, only a single CMV clone (2%) and 3 out of the 23 EF1a clones (13%) showed an MFI > 1000 a.u. Overall, the MFI was higher for the EF1a driven clones (Fig. 1D). In the 'low' expression group generated with the CMV construct, most clones showed expression between 100 and 1000 a.u., while the EF1a clones had lost expression (Fig. 1C, E and overall summary Fig. 2B).

Besides the variable expression level in the individual clones generated upon sorting (clone-to-clone variation), the analysis showed a pronounced intraclonal heterogeneity of both high and low sorted populations from the CMV clones and the high sort EF1a clones (see Fig. 1B–D). Some of the clonal populations displayed fractions of cells with low and high expression, respectively (e.g. CMV clones 19, 26 and 51 in Fig. 1B and most of EF1a clones of the high sort group, Fig. 1D). In other populations, a significant fraction of cells had lost expression completely (e.g. CMV clones 21, 39 (Fig. 1B) and EF1a clones 9 and 16 (Fig. 1D)). We even identified clones which showed complete lack of transgene expression at p2 (CMV clones 12, 49, 55 (Fig. 1B) and EF1a clones 13 and 23 (Fig. 1D)). Based on our handling protocols we excluded that GFP negative clones might result from a contamination with non-infected cells during sorting. Thus, the statistical evaluation of the 125 clones indicates heterogeneity and pronounced silencing of transgene expression already within the first generations after transgene integration in most of the cell clones.

3.2. Loss of transgene expression within first passages does not correlate to CpG methylation of the CMV promoter

We focussed our further studies on the 55 CMV clones from the 'high' expression cells characterized by MFI >1000. Of note, within

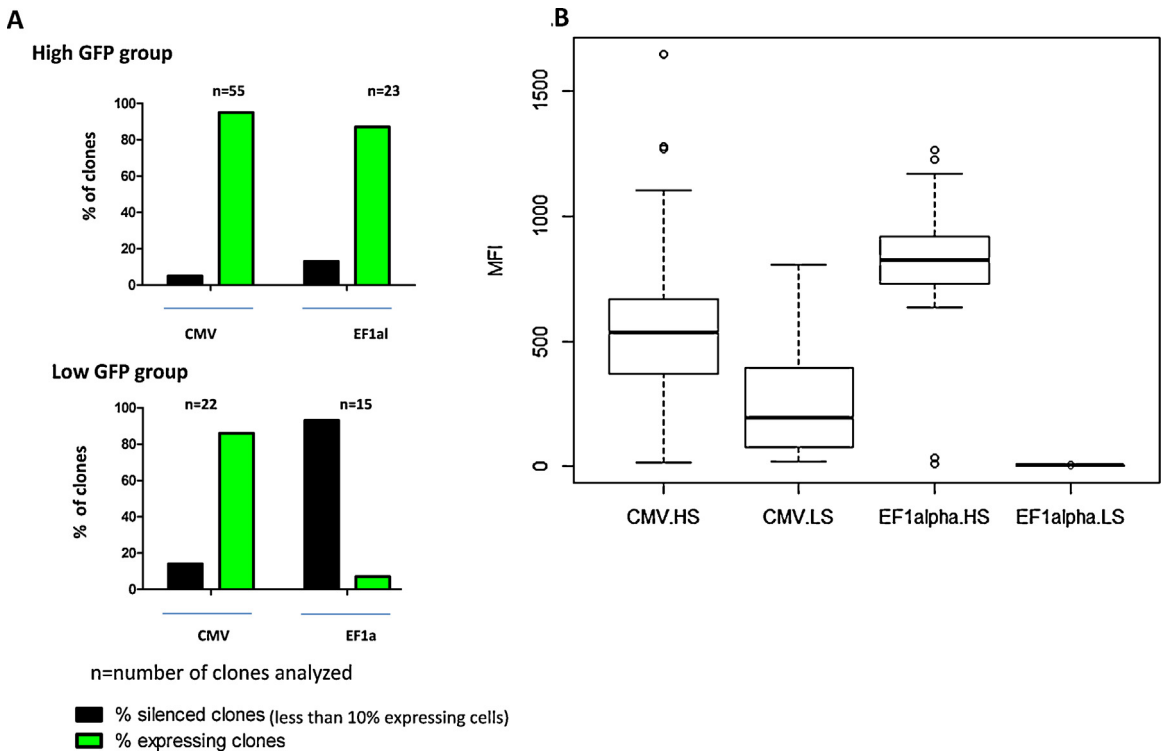


Fig. 2. Statistical evaluation of GFP expression of clones. (A) Statistical evaluation of GFP expression in CHOK1 cell clones generated from low (upper panel) and high (lower panel) expression single cells for the two promoters. Black and green bars indicate the percentage of clones showing less or more than 10% GFP expressing cells, respectively. (B) Box plot analysis comprising all 125 clones summarized in Fig. 1B–E. The median, the first and third quartile are indicated. Whiskers represent the maximum and minimum values excluding the outliers (Q1 − 1.5 × interquartile region, IQR or above Q3 + 1.5 × IQR).

the first 17 cell generations (p2), the expression of the 55 clones dropped to a mean MFI of about 500 a.u. (Fig. 3A). We hypothesized that the loss in transgene expression at this early stage of clone generation might be modulated by methylation of the CMV promoter. The CMV promoter – although providing high levels of expression in many cell types – has been shown to be sensitive to DNA methylation both in vivo and in vitro (Brooks et al., 2004; Mehta et al., 2009; Meilinger et al., 2009). The degree of methylation was found to correlate with the expression status of the cells (Yang et al., 2010). To characterize the methylation status of the CMV promoter in our setting, we performed bisulfite conversion of genomic DNA isolated from the clones 12 and 55 which completely lost transgene expression at p2 (Fig. 3B). The CMV promoter fragments were amplified by PCR and cloned in *Escherichia coli*. Eight bacterial clones from each cell clone reflecting the promoter sequence of an individual CHOK1 cell were randomly picked and sequenced. We focused our analysis on a 283 bp fragment of the CMV promoter encompassing the TATA box and essential transcription factor binding sites as well as 14 CpG sites which could be potentially methylated (Fig. S1) (Butta et al., 2006; Mancini et al., 1999; Tate and Bird, 1993). Interestingly, the sequencing revealed complete absence or rarely methylated CpGs for these homogenously silenced clones (Fig. 3B).

We further monitored expression of the GFP reporter in the 55 CHO clones upon cultivation for 11 passages corresponding to about 27 generations. Interestingly, we found some cell clones that showed significant alteration in expression of GFP during this time (13/55, data not shown) which was also reflected by a significant overall decrease in MFI during this time period (Fig. 3A). We asked if this change in expression was a consequence of DNA methylation that could affect the transgene or cellular genes crucial for expression. We subjected the 55 clones to treatment with Decitabine, a potent inhibitor of DNA methyltransferases (Dnmti). However, in all the clones the MFI only changed marginally and overall, the Decitabine induced change of MFI was not significant (Fig. 3A).

From these results we conclude that DNA methylation does neither cause nor correlate with the phenotypic heterogeneity and is thus not causative or related in early silencing of GFP expression in CHOK1 derived cell clones.

3.3. Cell to cell variability in clonal populations with unstable chromatin conformations

While during the cultivation for 11 passages most of the clones showed a homogenous and gradual decrease in expression, surprisingly, few clones displayed a more dynamic and stochastic expression phenotype. These clones were characterized by large variability in the GFP expression from cells of the same clone (intraclonal variations) with fractions of cell populations in GFP expressing and non-expressing states (see Fig. 4A for representative clones 25 and 35). We evaluated if the treatment of cells with inhibitors of histone deacetylases (HDAC) would allow to revert the silenced state of cells and/or to increase the transgene expression thereby making the clonal populations more homogeneous.

For this purpose we treated the 55 long-term cultured cell clones for 72 h with HDAC inhibitor sodium butyrate (NaB) (Mariani et al., 2003) or Valproic acid (VPA) and subsequently reanalyzed the cells for GFP expression. In many cell clones an increase of the expressing population was observed upon treatment with the HDAC inhibitors. Moreover, the overall expression (MFI) was dramatically increased (see Fig. 4A for selected NaB treated clones and S2A and S2B for overview on NaB or VPA treatment of all the 55 clones). Interestingly, the coefficient of variance, as given by the standard deviation

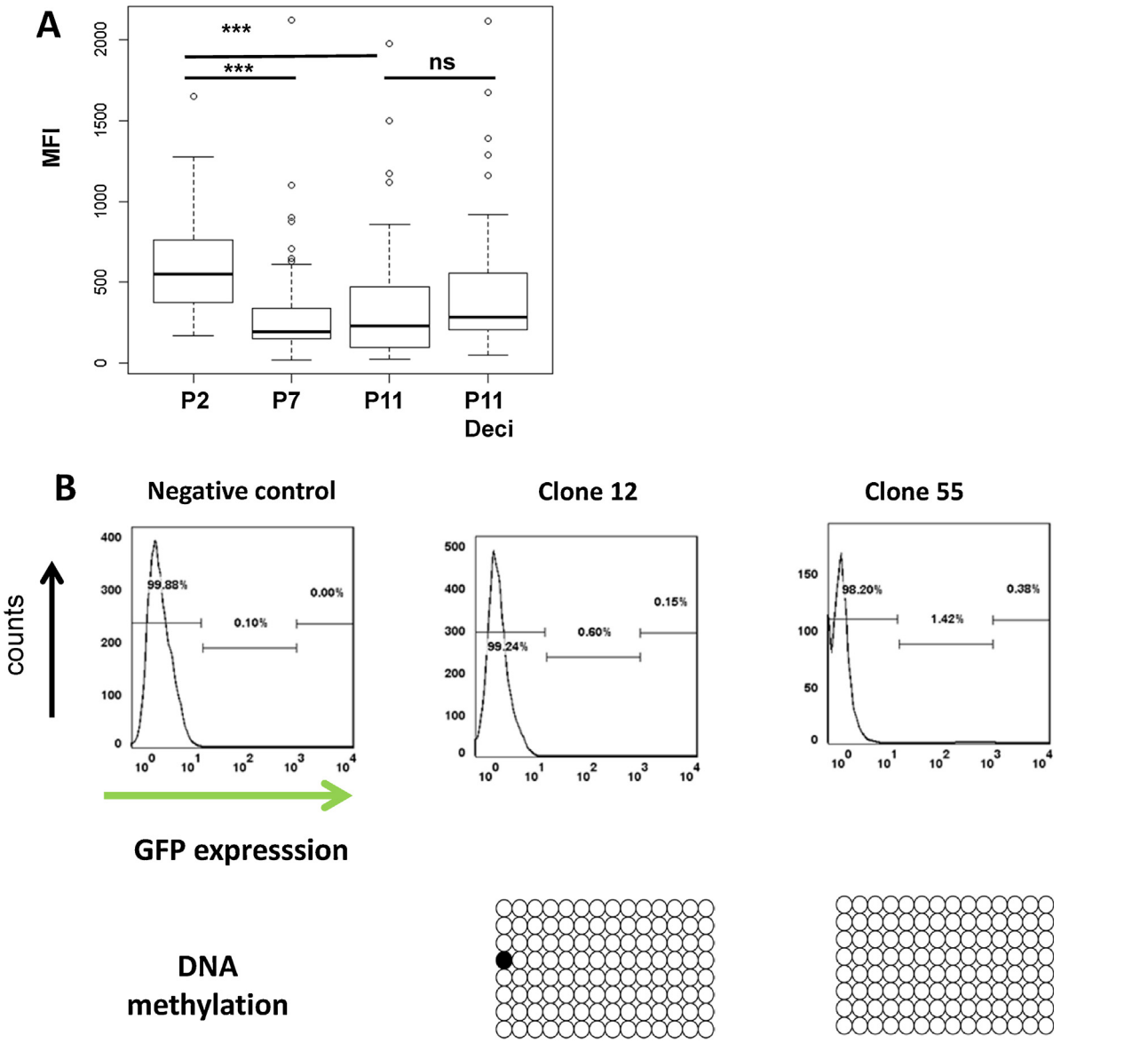


Fig. 3. Stability of gene expression in cell clones established from GFP high sort group. (A) Representation of overall expression level of the 55 clones at indicated passage numbers after sorting for high GFP expression. Box plots with the median as well as the upper and lower quartiles display GFP expression. Whiskers represent the maximum and minimum values excluding the outliers (Q1 − 1.5 × interquartile region, IQR or above Q3 + 1.5 × IQR). *** $p < 0.001$; ns $p > 0.05$. Deci indicates the expression of p11 cell clones after treatment with Decitabine. (B) The FACS plots display the GFP levels of CMV clones 12 and 55 (p2) that were established from high GFP expressing cells. The lower panel represents the DNA methylation status of the CMV promoter analyzed upon bisulfite conversion. Each circle in a line represents a CpG dinucleotide of a single PCR fragment according to the sequence indicated in Fig. S2. Unfilled circle represent non-methylated CpG dinucleotides and black filled circles represent methylated CpG dinucleotides. Results from 8 fragments representing 8 individual cells of a clonal population are represented.

of expression divided by the mean, decreased within clones upon treatment. Thus, expression of the clonal populations became more uniform (Fig. 4B). Of note, some cell clones remained completely unaffected and did not show any changes in the expression pattern (exemplified by clones 12 and 49).

We asked if the induced changes were stably inherited upon further cultivation of cells. Thus, we reanalyzed the NaB responsive clones 2, 9, 10 and 17 after further six passages in absence of the inhibitor. Surprisingly, the NaB induced increase in expression completely reverted in the absence of the drug, indicating that the changes are not stably inherited (Fig. 5). Interestingly, transgene expression decreased even below the expression level of untreated cell clones passaged for the same times (see clones 10 and 17 in Fig. 5). Thus, this suggests that the treatment results in destabilizing of the chromatin.

3.4. Influence of chromosomal environment is inherited upon exchange of expression cassettes

Chromosomal sites can be exploited by targeted integration of expression cassettes of choice (Coroadinha et al., 2006; Gama-Norton et al., 2011; Kunert and Casanova, 2013; Nehlsen et al., 2009; Ou et al., 2009; Schucht et al., 2006) We asked if the modulation of transgene expression at a particular site is a specific feature of the chromosomal locus and would be transferred also to incoming 'naked' DNA upon targeting. We evaluated this potential in the CHOK1 clones 9 and 51 which showed homogenous and high expression during the 11 passages monitored (data not shown and Fig. 6). For this purpose we used the FRT-WT and FRT-F5 sequences for integrating a naive expression cassette by recombinase mediated cassette exchange (RMCE).

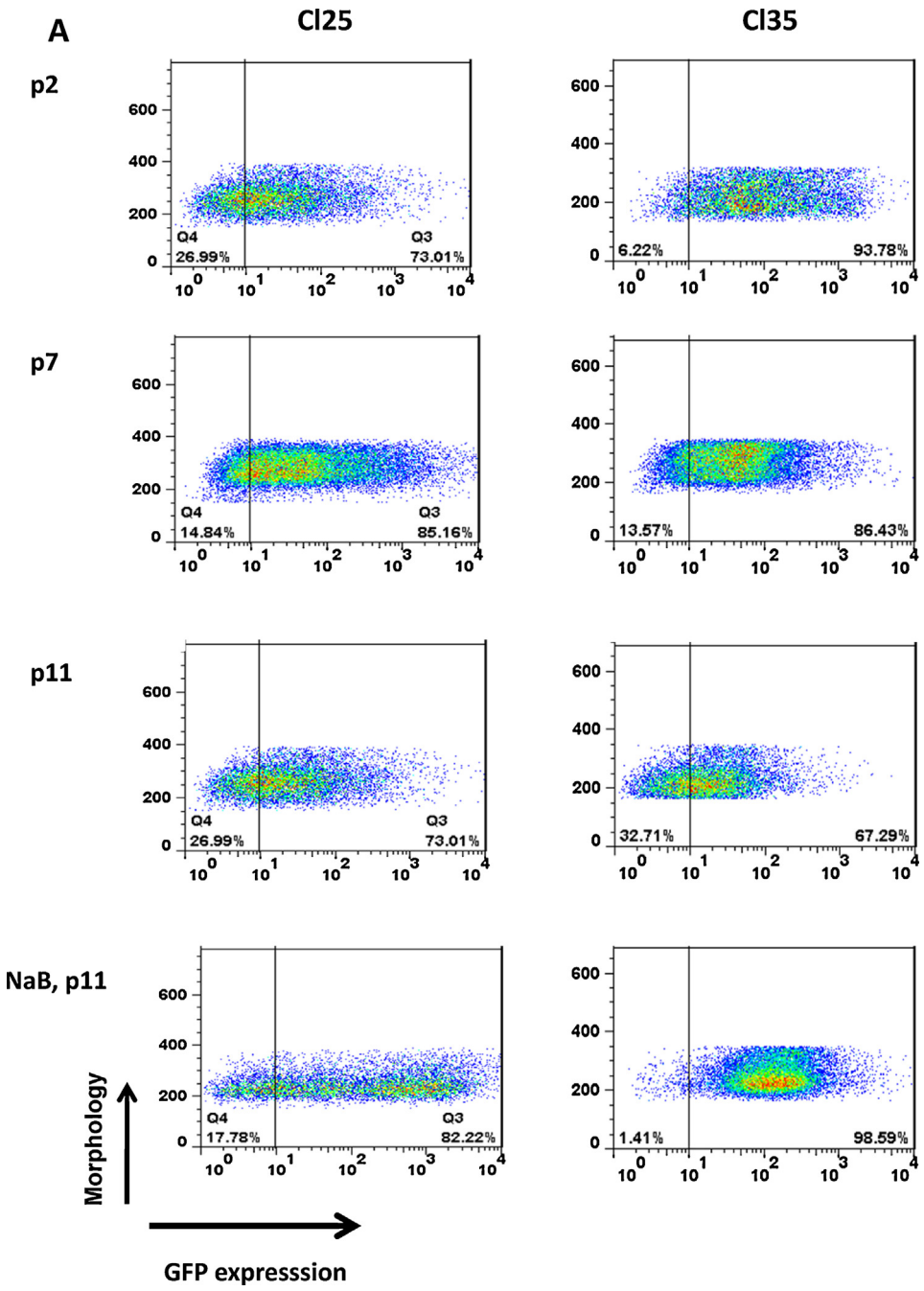


Fig. 4. Dynamics of GFP expression upon clone expansion. (A) Flow cytometry was used to follow expression of clones 25 and 35 upon long term cultivation and upon treatment with the HDAC inhibitor NaB at p11. (B) The graph depicts the mean fluorescent intensity (MFI) and coefficient of variance (CV) of cell populations shown in A.

The cell populations harbouring CMV-GFP cassettes were targeted with an FRT-WT/F5 flanked cassette comprising the CMV promoter or the SV40 promoter driving RFP (see Fig. 1A). As a consequence of Flp mediated cassette exchange, targeted cells lose GFP expression and can be isolated by FACS, thereby avoiding any selection regimen that could potentially induce epigenetic alterations.

As expected, transfection of the recombinase plasmid pFlpe alone did not alter the percentage of GFP expressing cells (Fig. 6). This indicates that the GFP cassette is not lost upon transfection/expression of FlpE. However, upon transfecting the recombinase vector together with the targeting vector, a fraction of cells lost GFP indicating that these cells RMCE have undergone RMCE. These cells were isolated via sorting, expanded for two passages and confirmed for targeted integration through PCR (data not shown).

Upon targeting clones 9 and 51 with the CMV-RFP cassette, nearly all the sorted cell populations showed a high and homogeneous RFP expression, comparable to the GFP expression in the respective parental cells. This indicates that the specific influence of the chromosomal site on the transgenic CMV promoter element is re-established upon integration of the epigenetically 'neutral' plasmid sequence.

Upon integration of the SV40-RFP cassette, we observed a differential outcome in the two cell populations. For clone 9 the expression phenotype of the parental population was maintained

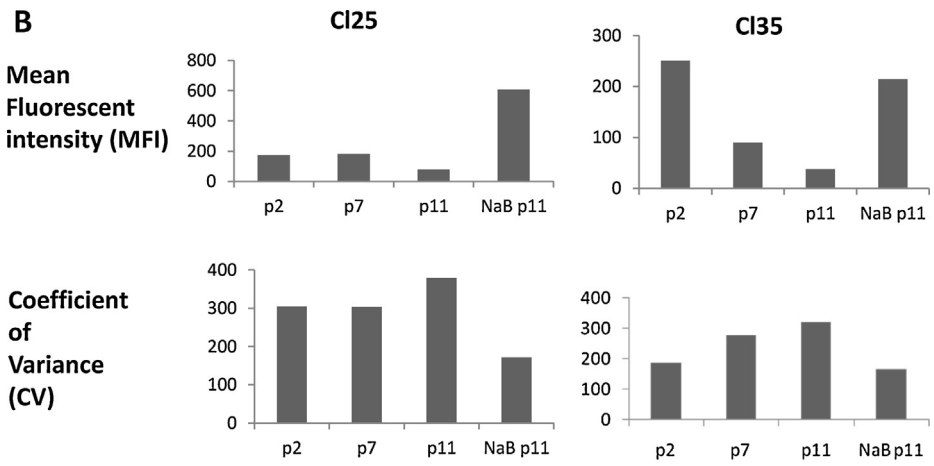


Fig. 4. (*Contnued*)

but was reduced. Strikingly, a highly heterogeneous expression was observed for clone 51 with a significant fraction of cells not expressing RFP although being successfully targeted (Fig. 6).

We asked which mechanism could be responsible for silencing of the SV40 promoter in cells from clone 51. We investigated if methylation set in after targeting of the SV40 promoter cassette and contributed to the partial loss of expression. To this end, the non-expressing and expressing cell fraction of SV40-RFP targeted clone 51 were sorted and subjected to DNA methylation analysis of the SV40 promoter. Interestingly, from the 14 CpGs of the SV40 promoter evaluated, none of them was affected by DNA methylation (Fig. S4A). Interestingly, also upon treatment of the cell population with HDAC inhibitors NaB and VPA no significant change in expression was observed (Fig. S4B). This suggests that the SV40 promoter is not modulated by DNA methylation or histone acetylation in these clones.

Together, this indicates that homogenous transgene expression within a specific chromosomal site can become heterogeneous when integrating a different promoter element. Moreover, the data suggest that chromosomal integration sites can differentially modulate various incoming promoter elements indicating that the epigenetic pattern is governed by a specific crosstalk of the promoter and the integration site.

4. Discussion

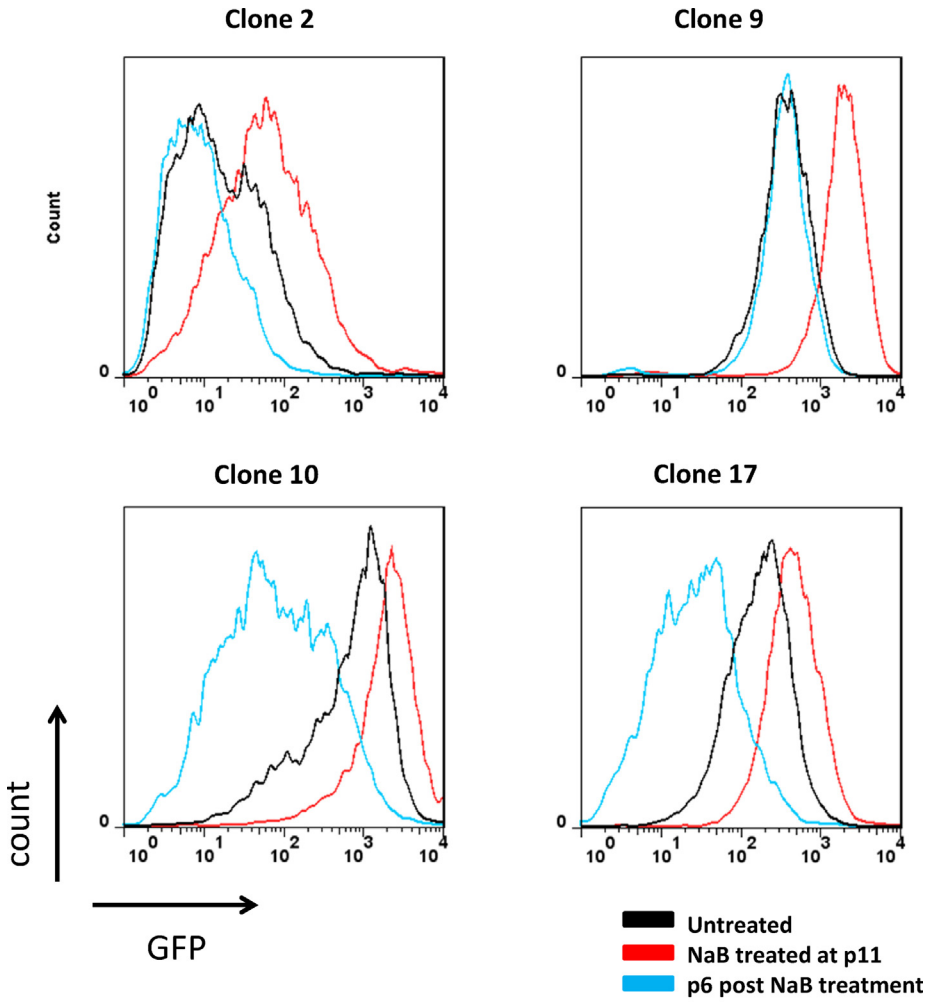
In this study, we investigated the stability of transgene expression in CHOK1 clones that were established upon random integration of a single copy transgene expression cassette in absence of selection. We isolated individual cells that displayed a high or low expression level and established clones thereoff. Already at p2 we observed reduced overall GFP expression and pronounced to cell-to-cell (intraclonal) variation in most of the clones. This was observed both for the EF1a and the CMV promoter based clones. Of note, in our study we used the rather stable GFP protein as a reporter (half-life of 26 h (Corish and Tyler-Smith, 1999)). Thus, we can exclude that the variations observed upon establishment of clones arose from bursts of expression that can be a cause of intraclonal expression variations observed for short-lived proteins (Dar et al., 2012; Raj et al., 2006; Smith and Workman, 2012).

The instability of transgene expression was most striking in clones that showed complete loss of transgene expression at p2 although being derived from cells that showed high GFP expression at p0. This shows that such a dramatic modulation of expression can be homogeneously manifested within the first 17 cell generations after integration of the transgene cassette. Of note, this early phase in transgene silencing upon chromosomal integration is masked when applying standard protocols for clone generation that rely on selection regimens.

To identify the epigenetic mechanism underlying loss of transgene expression and causing this heterogeneity, we evaluated the impact of DNA methylation. Methylated DNA has been shown to be associated to loss of transgene expression and also can convey fast changes in expression (He et al., 2005). Surprisingly, in two clones that completely switched off transgene expression we excluded that DNA methylation of the promoter was the underlying mechanism of silencing (Fig. 3B). This was unexpected given the fact that the CMV promoter is a frequent target for DNA methylation (Meilinger et al., 2009; Osterlehner et al., 2011; Yang et al., 2010). We cultivated the cells in presence of Decitabine, a widely used inhibitor of DNA methyl transferases (DNMTs). This nucleoside analogue is phosphorylated in the cells and incorporates into the DNA. Decitabine covalently binds to the DNMTs and with repeated divisions DNMTs are depleted which results in significant DNA demethylation (Lubbert, 2000). However, treatment with Decitabine did not increase the expression level (Fig. 3A and data not shown). This excludes that methylation of cellular genes crucial for transgene expression is the cause for the loss of transgene expression.

Histone modifications have been considered to convey dynamic changes that can be triggered, e.g., by slight change in the environment (Smith and Workman, 2012; Weiner et al., 2012). Decrease in histone acetylation can lead to a more compact status of the DNA: the acetyl groups can neutralize the positive charges of histones whereby interaction between histone and negatively charged phosphate group of DNA is impaired (Garcia-Ramirez et al., 1995). The degree of histone acetylation can be modulated by inhibitors of histone acetylation (HDACi). Most of the HDACi used today have Zn^{++} chelating groups. These groups can fit into the active pockets of HDAC from class I, II and IV and disrupt the formation of these complexes (de Ruijter et al., 2003; Delcuve et al., 2012, 2013)

Strikingly, treatment with the epigenetic modifiers VPA and NaB could increase transgene expression in many clones. This suggests that the chromatin status at the site of transgene integration was characterized by suboptimal levels of acetylation which rendered the transgene cassette in a less favourable state for transcription.



Overall expression level (MFI)

	Clone 2	Clone9	Clone10	Clone17
untreated cells	35	422	938	214
NaB treated	144	2024	2240	537
P6 post NaB treatment	17	387	203	52

Fig. 5. Transgene expression after HDAC inhibition. The indicated cell clones were treated with NaB at p11 and analyzed for expression (red lines). The clones were cultivated for further 6 passages in absence of NaB and reanalyzed at p17 (blue lines). Untreated cells (p17) are represented by black lines. The mean fluorescent intensities (MFI) from the presented FACS plots are indicated in the table.

However, since these inhibitors may also act through inhibition of deacetylation of non-histone protein targets we cannot rule out that the epigenetic modulation of cellular genes is indirect. The fact that expression is restored in many clones suggests that the final target is the promoter activity. Further in-depth characterization of histone modifications both at the transgene and on cellular genes would be required to elucidate this point. This is currently hampered due to lack of good and robust characterization methods for hamster cells.

The effect of the inhibition of HDACs was transient and transgene expression dropped upon withdrawal of the chemical inhibitors. Thus, this activated state was unstable and continuous acetylation would be required to maintain the expression phenotype in these cell clones. Importantly, the restoration of expression after application of histone modifiers also excludes that loss of gene expression in these clones is a consequence of genetic rearrangements, deletions or mutations. Interestingly, after one round of HDAC inhibition a decrease in transgene expression was observed in some clones, suggesting that the inhibitor induced modulation of the histone state per se may cause a pronounced instability at the transgene integration site. We cannot rule out that this observation arises as a consequence of acetylation independent off-target effects induced by the inhibitors. However, the fact that in most clones both NaB and VPA induced similar effects argues against this possibility.

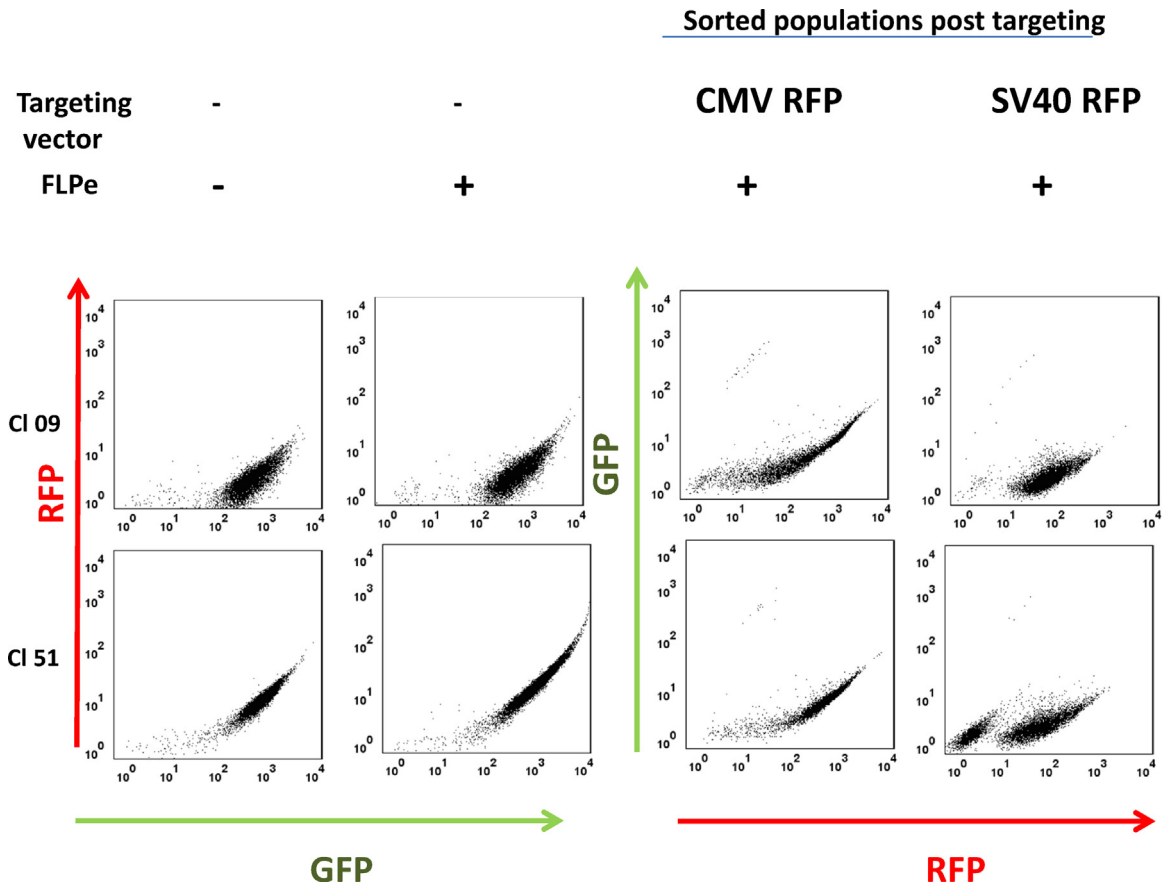


Fig. 6. Expression profiles upon exchange of expression cassettes. Clones 9 and 51 were subjected to exchange of expression cassettes with a CMV-RFP and SV40-RFP cassette as specified in the text. Correctly targeted cells were isolated by sorting for loss of GFP expression (see Fig. S4 for details of the sorting strategy). The right two panels show RFP expression of sorted cells. Non-transfected parental cells as well as cells only transfected with Flpe recombinase are shown as controls (left panel). The figures are representative for two independent experiments.

Recently, highly dynamic modulation of transgene expression resulting in intraclonal diversity was demonstrated for a particular CHO clone (Pilbrough et al., 2009). However, it remains unclear if this observation is a consequence of copy number mediated modulation of transgene expression and/or if this is linked to a particular chromosomal site. Here, we show that intraclonal alteration of transgene expression can occur in many different chromosomal integration sites even upon single copy integration suggesting that this is a common property.

Together, the results suggest that in most of the cell clones the epigenetic silencing during the early phase of clone generation is governed by histone modification. The fact that in our setting loss of transgene expression is not associated to DNA methylation seems to be in contrast to previously published data might be explained by the experimental features of the current study:

(i) We identified the cells upon lentiviral gene transfer. Theoretically, this might enrich integration sites that are less prone to DNA methylation (Pfeifer et al., 2002). Still, this reflects many sites in the genome that are also accessible upon non-viral gene transfer.
(ii) We focused on single copy integrations. Thus, we can attribute the observed heterogeneity to a specific integration site. This excludes silencing initiated by integration of multiple copies of transgenes at a particular site which was previously reported to accompany tandem or multi-copy integrations (Ellis, 2005; Eszterhas et al., 2002; McBurney et al., 2002).
(iii) We monitored cell clones within the first 11 passages after recombinant DNA integration which is shorter than the analysis of silencing that reported CMV promoter linked methylation. Thus, we cannot exclude that histone mediated silencing may be followed by DNA methylation. Indeed, some studies show that upon long term cultivation of CHO cells, DNA methylation can set in (Kim et al., 2011; Osterlehner et al., 2011).

Targeting of pre-selected chromosomal sites has been proposed to overcome position dependent expression of heterologous expression. Indeed, this method allows predicting expression of transgenes and thereby facilitates fast and reliable establishment of production cell lines (Gama-Norton et al., 2011; Schucht et al., 2006; Wong et al., 2005). Here, we employed this method to investigate if an expression pattern is re-established on a neutral cassette upon targeted integration of plasmid DNA. We decided to rely on a bias-free approach and isolated the targeted clones by sorting for excision of the tagging cassette. Importantly, this avoided any selection for expression of a resistance marker which might impose per se alterations in the epigenetic pattern of the incoming cassette. Interestingly, by re-integration of the epigenetically neutral CMV promoter driving RFP as a reporter we could re-establish and restore the phenotype of the parental cells.

In contrast, when we targeted the SV40 promoter driving same reporter the predictability in the expression was lost. Only in clone 9 the expression status could be reproduced upon cassette

exchange. Clone 51 displayed a complex expression pattern upon targeting: a fraction of cells expressed at high levels, while the other cells completely lost expression (Fig. 6). Of note, loss of expression after cassette exchange was not accompanied by methylation of the promoter DNA, the most frequently observed mechanism of silencing. Interestingly, the SV40 promoter was devoid of CpG methylation when targeted in clone 51 suggesting that this mechanism is not governing silencing (in case of this promoter). In contrast to the parental CMV clone 51, the SV40 targeted clone 51 was not susceptible to treatment with the HDAC inhibitors NaB or VPA. This strongly suggests that another epigenetic modification governs the chromatin status in this particular chromosomal site. Alternatively, methylation of cellular genes that are essential for expressing the SV40 RFP cassette could be affected. Overall, the phenotype of the cell population after targeting with SV40-RFP is interesting since it displays a strictly bimodal (on/off) state. Such a bimodal expression pattern has been previously described both for endogenous genes such as IRF-7 (Rand et al., 2012) and synthetic autoregulated cassettes (May et al., 2008). In the SV40-RFP pools derived from RMCE the bimodal expression state suggests the establishment of two distinct chromatin states that are established after targeting. It is tempting to speculate that these states are established by defined epigenetic histone modifications.

The results support the previously highlighted differential influence of a given chromosomal integration site on incoming promoters in CHOK1 cells (Nehlsen et al., 2009, 2011). Moreover, this study could define the mechanism underlying differential crosstalk. Of note, similar results were obtained when studying expression upon RMCE in 293T cells (data not shown) suggesting that this is a general feature of transgene expression.

It is tempting to speculate about the expression upon targeting epigenetically silenced, non-expressing chromosomal loci. However, there are some reports that recombination in heterochromatic regions is less favoured both for cellular recombination and site specific recombinases (Ahmad and Golic, 1996; Blumenstiel et al., 2002; Long and Rossi, 2009; Mostoslavsky et al., 2003) which makes silent loci less available by such targeting approaches. Moreover, identification of recombined cells in absence of expression is difficult due to the unavailability of efficient methods identifying targeted non-expressing clones.

Together, this study sheds light on the epigenetic modulation of transgenes immediately upon integration into CHOK1 cells. Our findings that histone regulation is involved in intraclonal variation suggest an epigenetic crosstalk between the incoming cassette and the integration site. We propose that stochastic silencing mediated by histone modifications is an inherent property of the integration site which is re-established upon integration of transgene cassettes having an isogenic promoter region. We anticipate that this will have important implications for the exploitation of chromosomal sites upon integration of transgenes both in vitro and in transgenic animals. Finally, the variability of transgene expression upon chromosomal integration also mimics the modulation of endogenous genes once they are exposed to an artificial chromosomal context. This situation can be also naturally established, e.g., upon translocations and rearrangement in cancer cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.12.009.

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