Journal of Genetics and Genomics 43 (2016) 503–511

Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Original research

Epigenetic modulations rendering cell-to-cell variability and phenotypic metastability

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ARTICLE INFO

Article history:
Received 8 January 2016
Received in revised form
12 May 2016
Accepted 25 May 2016
Available online 27 May 2016

Keywords:
Intraclonal heterogeneity
Epigenetic regulation
DNA methylation
Differential histone marks
Metastable expression

ABSTRACT

Tumor cells display phenotypic plasticity and heterogeneity due to genetic and epigenetic variations which limit the predictability of therapeutic interventions. Chromatin modifications can arise stochastically but can also be a consequence of environmental influences such as the microenvironment of cancer cells. A better understanding of the impact and dynamics of epigenetic modulation at defined chromosomal sites is required to get access to the underlying mechanisms. We investigated the epigenetic modulations leading to cell-to-cell heterogeneity in a tumor cell line model. To this end, we analyzed expression variance in 80 genetically uniform cell populations having a single-copy reporter randomly integrated in the genome. Single-cell analysis showed high intraclonal heterogeneity. Epigenetic characterization revealed that expression heterogeneity was accompanied by differential histone marks whereas contribution of DNA methylation could be excluded. Strikingly, some clones revealed a highly dynamic, stochastically altered chromatin state of the transgene cassette which was accompanied with a metastable expression pattern. In contrast, other clones represented a robust chromatin state of the transgene cassette with a stable expression pattern. Together, these results elucidate locus-specific epigenetic modulation in gene expression that contributes to phenotypic heterogeneity of cells and might account for cellular plasticity.

1. Introduction

The heterogeneity and cellular plasticity observed in cancer cell populations represent a major hurdle in treating cancer patients. The development of resistance in the metastatic cells limits the utility of the therapeutic remedies. While most of this heterogeneity was previously thought to be due to genetic alterations and inherent genetic instability of cancer cells (Marusyk et al., 2012), there is increasing evidence showing that genetic mutations cannot be held as a sole cause of this heterogeneity (Marjanovic et al., 2013). Studies have shown that the disruption in the epigenetic marks can also be an important intrinsic factor that might result in cellular heterogeneity and plasticity (Huang, 2013; Marjanovic et al., 2013).

The phenotypic reversibility and metastability frequently observed in tumor populations are considered to be significantly contributed by dynamic chromatin markings (Huang, 2013; Marjanovic et al., 2013). These structures might act as sensors and effectors (mediators) to adjust the selection pressure exerted by the cellular microenvironment.

Epigenetic modifications are known to critically affect the chromatin state. This includes changes in the methylation pattern of DNA as well as specific histone modifications such as methylation and acetylation on the specific amino acid residues of histones (Ghavifekr Fakhr et al., 2013). Thereby, the differential accessibility and/or binding of DNA sequences by a set of proteins are realized. Together, this modulates the efficiency of transcription and as a consequence the cellular phenotype (Cui et al., 2013; Li, 2013; Buck et al., 2014). 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

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http://dx.doi.org/10.1016/j.jgg.2016.05.008

and acetylation that can occur on specific residues (e.g., lysine) 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. In recent times, a large number of studies have permitted a partial unraveling of this code (Misri et al., 2008; Gacek and Strauss, 2012). 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 were shown to be associated with active gene expression (Kirmizis et al., 2004; Yu et al., 2007; Pauler et al., 2009; Connolly et al., 2013).

Most of our understanding of the role of epigenetics in cancer is based on studies of differential expression of cellular oncogenes and tumor suppressor genes in their natural chromosomal context. However, tumor progression is frequently accompanied with an inherent genomic instability. As a result of genomic rearrangements, deletions and translocations can occur. As a consequence, genes are subjected to influences arising from new genetic environments.

We aimed at a better understanding of the phenotypic variation of gene expression that may occur if genes are exposed to novel chromosomal environments. To simulate this situation, we investigated the epigenetic mechanism(s) underlying the alterations in expression of single-copy transgenes randomly integrated into chromosomal sites of a tumor cell line without selection pressure. Interestingly, we could correlate the expression phenotype with defined histone modifications. Depending on the particular chromosomal site, these chromatin modifications were either stable or dynamically changed upon prolonged cultivation. Together, the results highlight the plasticity of chromatin modulation upon rearrangement and resulting phenotypic variations in cancer cells.

2. Results

2.1. Expression heterogeneity in HEK293T clones with a single-copy GFP expression cassette

To establish an *in vitro* system to study the mechanism(s) that cause epigenetically mediated variation in gene expression, we used genetically stable single-copy transgenes as sentinels. To simulate the influence of epigenetic variations in different chromosomal sites, we analyzed randomly chosen integration sites of a sentinel transgene. We employed SV40 T antigen-transfected human HEK293T cells which represent a model for cancer stem cells (Debeb et al., 2010). To set up a strategy to identify chromosomal sites that support transgene expression, we employed a transgene screening cassette comprising the human cytomegalovirus (CMV) promoter that drives a reporter gene encoding a stable GFP protein (half-life >20 h (Corish and Tyler-Smith, 1999)). This promoter was shown to be susceptible to epigenetic modifications (Grassi et al., 2003; Mehta et al., 2009; Hsu et al., 2010). 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′ long terminal repeat (LTR) was employed to avoid interference of the viral regulatory elements with the CMV promoter upon infection (Fig. S1). To ensure single-copy integration of the screening cassette, infection was performed at a multiplicity of infection (MOI) of 0.01 using a standardized protocol. Thereby, statistically, 99% of expressing cells carry a single-copy integration of the expression cassette; in previous studies, we confirmed this protocol with respect to the efficient generation of single copy integrations (see Materials and methods for further details) (Schucht et al., 2006; Gama-Norton et al., 2011). Ten days after lentiviral infection, single cells with high (>10^3 arbitrary units (a.u.)) and low (10^1–10^3 a.u.) GFP fluorescence were sorted by FACS and clonally expanded. This state was defined as passage 0. At passage 2 after sorting, flow cytometry analysis was performed for 55 and 25 clones that had been established from the high and low GFP expressing population, respectively. At this time point, the clonal cells were expanded about 100,000 folds corresponding to about 17 generations. Such cell clones represent sentinel genes whose expression is dominated by the respective chromosomal neighborhoods.

The FACS analysis revealed large differences in GFP expression in the individual clonal cell populations. None of the 25 cell clones established from the cells sorted for low-level expression showed GFP expression at this time point (less than 0.4% expressing cells, data not shown). Cell clones established from the 55 high GFP expressing cells showed variable expression with high clone-to-clone variation (Fig. S2 for overview and Fig. 1 for details of representative clones). One of the clones (clone 42^T) even showed a dramatically decreased expression. We observed variable mean expression levels and a high intraclonal variation of expression in individual clones. Some HEK293T clones (e.g., clone 12^T and 35^T) showed a more homogeneous expression phenotype while others (e.g., clones 31^T and 42^T) displayed a pronounced variation of expression.

For further in-depth characterization, we selected five HEK293T clones with different levels of heterogeneity (12^T, 17^T, 31^T, 42^T and 54^T). In all of the clones, a distinct population of low/non-expressing cells was detected (Fig. 1). To separate GFP positive expressing (PS) and GFP non-expressing cells (NS) from these five clonal populations, cells were sorted at passage 3 after infection which corresponds about 20 cell generations (Fig. 2A for overall scheme and Fig. S3 for sorting details). To exclude that non-expressing cells were a result of contamination by non-transgenic cells, genomic DNA was isolated from the five NS populations. PCR was used to confirm transgene integration for all populations (data not shown).

To evaluate the stability of the expression phenotype of the sorted populations, the selected cell clones were expanded for further 25 passages (corresponding to a total of about 110 cell generations) and re-analyzed for respective expression. The subpopulations of clones 12^T, 17^T and 54^T showed a stable phenotype upon extended cultivation: the PS populations remained positive and the NS populations also remained negative for GFP (Fig. 2B). In contrast, the subpopulations of clones 31^T and 42^T changed their phenotype: the NS populations of these clones shifted towards higher expression levels, while the PS populations showed a partial loss of GFP expression. As a result, the respective populations partially merged. Thus, these cell clones undergo a continuous modulation of the phenotype from the non-expressing state to the expressing state and *vice versa*, thereby exhibiting a highly dynamic, metastable phenotypic state.

2.2. Intraclonal heterogeneity is not correlated to differential CpG methylation

Phenotypic loss of expression has been frequently associated with a high degree of DNA methylation in CpG islands (Esteller, 2002; Cohen et al., 2008; Kaise et al., 2008; Liu et al., 2010; Tahara et al., 2010). Thus, we hypothesized that the heterogeneity in transgene expression might be modulated by epigenetic modification of the promoter sequence. We analyzed the DNA methylation status in the NS and PS populations immediately after second sorting (passage 3). In particular, we focused our analysis on a 283-bp fragment of the CMV promoter encompassing the TATA box and essential transcription factor binding

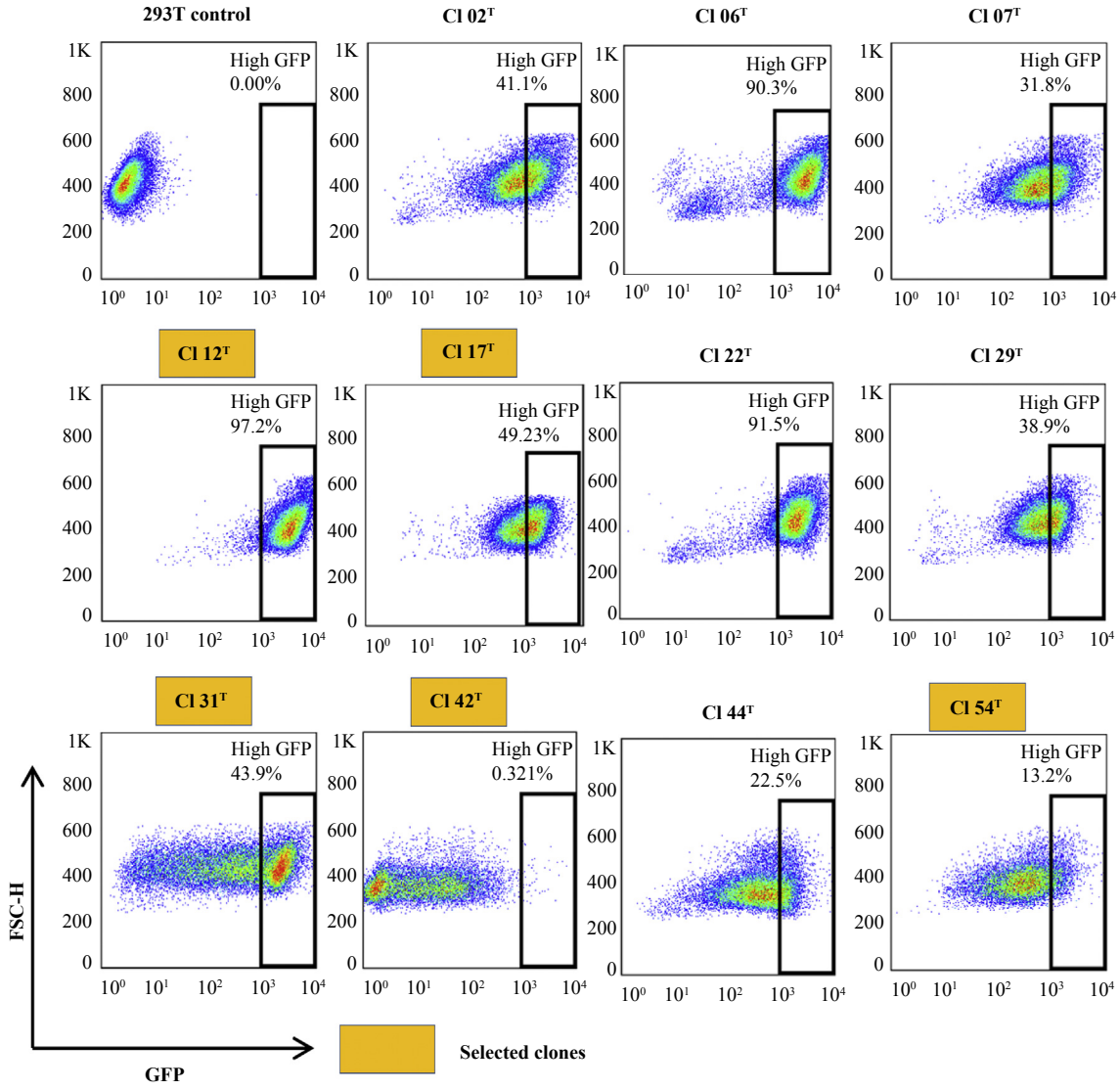

Fig. 1. Intraclonal heterogeneity in GFP expression in randomly selected chromosomal sites. FACS plots showing the GFP expression profiles of randomly selected HEK293T clones two passages after initial sorting for high expression GFP. The five selected clones from HEK293T for further characterization are marked by yellow rectangle.

sites as well as 14 CpG sites which could be potentially methylated (Fig. 3) (Tate and Bird, 1993; Mancini et al., 1999; Butta et al., 2006).

We performed bisulfite conversion of genomic DNA isolated from the NS and PS populations from the five clones. The CMV promoter fragment was amplified by PCR and cloned. Eight bacterial clones from each HEK293T cell clone reflecting the promoter sequence of an individual cell were randomly picked and sequenced. Unexpectedly, sequencing of the clones revealed C–T conversions for nearly all CpGs and thus complete absence or only rarely methylated CpGs, even for the NS populations (Fig. 3). The methylation status in these clones was also analyzed 40 passages post sorting. Importantly, also at this late time point, both the expressing and non-expressing cells remained largely free of CMV promoter methylation (data not shown). This excludes a delayed manifestation of DNA methylation upon prolonged passaging as previously suggested for other experimental settings (Jaenisch and Bird, 2003; Mutskov and Felsenfeld, 2004; Strunnikova et al., 2005). Thus, silencing of gene expression in HEK293T cells and intraclonal phenotypic variation are not reflected by differential methylation of the promoter.

2.3. Phenotypic variability correlates with differential histones markings

Since DNA methylation could be excluded as the underlying mechanism of phenotype variation in the subclonal populations, we analyzed the prevalence of differential histone modifications in the PS and NS subpopulations of the five selected clones. For this purpose, we performed chromatin immunoprecipitation (ChIP) and determined histone H3 lysine 4 acetylation marking (H3K4ac). H3K4ac is an abundant modification of transcriptionally active chromatin (Guillemette et al., 2011). The H3K4ac markings associated with the CMV promoter were quantified by PCR. As endogenous controls for active and repressed genes, we used previously validated primers for endogenous *MYT1* and *ACTB* genes, respectively (see Materials and methods for details). Interestingly, the cell populations of the five clones displayed a differential pattern of histone modifications. The PS populations (sorted for GFP expression) were enriched for the H3K4ac marking. In contrast, the NS population (GFP negative) showed these H3K4ac marks with much less frequency (Fig. 4). This indicates that the expression phenotype correlates with elevated levels of H3K4ac.

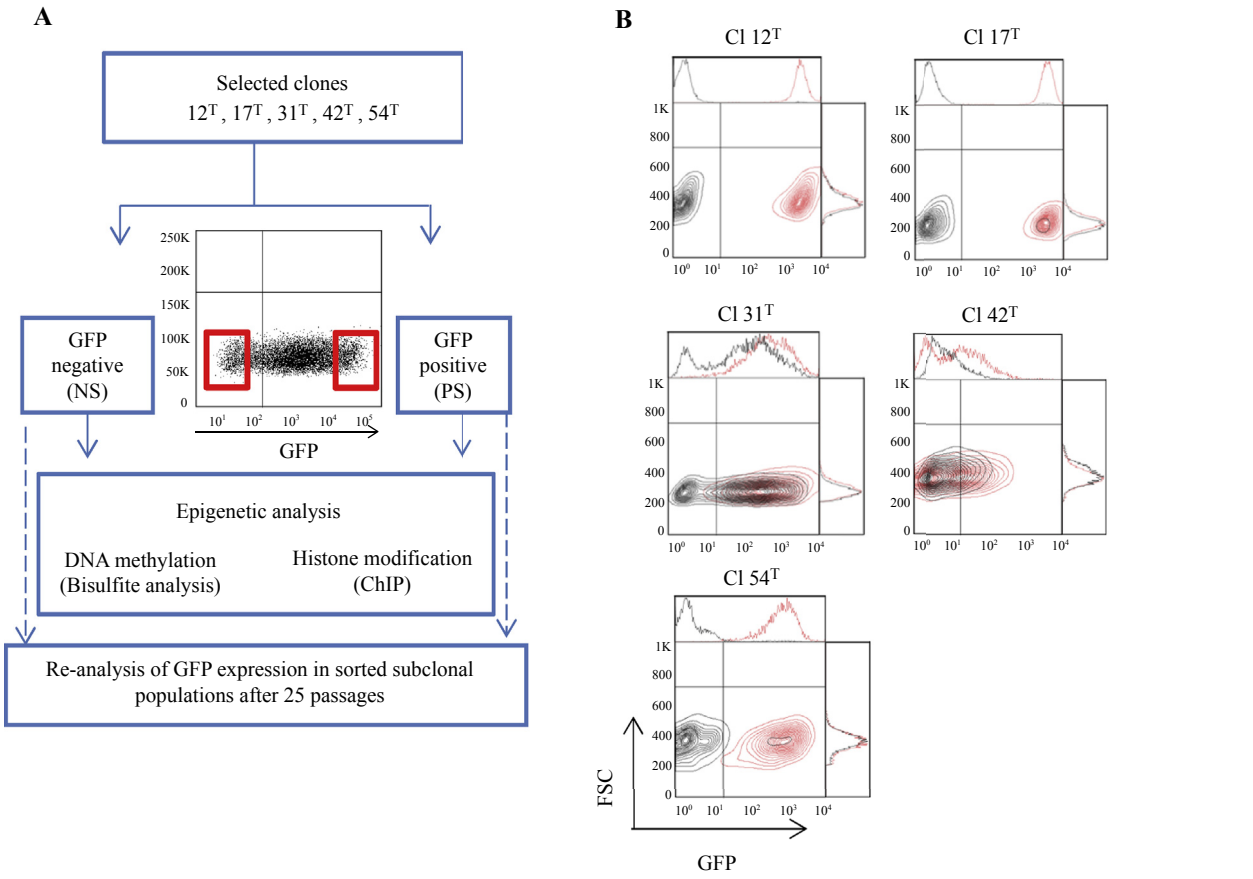

Fig. 2. Expression stability upon prolonged cultivation. **A**: Overview of the scheme for in-depth characterization of five selected clones from HEK293T cells. **B**: NS and PS subpopulations were generated from the five selected clones (see Fig. S2 for sorting details). The subpopulations were cultivated for 25 passages after initial sorting and were re-analyzed for GFP expression by flow cytometry. The plots represent the merged expression profiles of the subpopulations (black line: NS; red line: PS).

We then looked for the trimethylation of lysine 27 on the histone H3 (H3K27me3) which is known not only to suppress transcription in a DNA methylation independent manner but also to be responsible for stabilizing the silenced phenotype (Angel et al., 2011). We observed a strong enrichment of the H3K27me3 marking in the CMV promoter of the NS populations of all clones in comparison to the PS population with GFP expressing cells (Fig. 4). These results show that the phenotype of the sorted populations correlates with a differential histone modification pattern of the reporter.

2.4. Dynamic histone modifications: characteristics of "metastable" phenotypes

Since histone modifications correlate with the phenotypic changes in the clonal populations, we speculated that treatment of non-expressing cell populations with inhibitors of histone deacetylation (HDACi) is able to revert the silenced state and to increase transgene expression. For this purpose, we used HDACi sodium butyrate (NaB) (Mariani et al., 2003) and Valproic acid (VPA) (Wulhfard et al., 2010; Boudadi et al., 2013). Interestingly, the five cell clones responded differentially to the treatment with these drugs. For clones 31^T and 42^T, these drugs induced significant increases in the mean fluorescent intensity and in the percentage of expressing population (Fig. 5A and B). This indicates that the histone marks were dynamic in these populations and could be altered with the treatment. However, there were only slight increase of the mean fluorescence intensity and the percentage of GFP expressing cells in the NS population of clone 54^T, and the NS populations of clones 12^T and 17^T did not significantly revert to the expressing state (Fig. S4). Of note, when the negative sorted populations of these clones were subjected to Decitabine, an inhibitor of DNA methylation, we did not observe any change in *GFP* gene expression while endogenous control genes were up-regulated in these conditions (Fig. S5). This suggests that these populations are locked in an epigenetically silenced state and cannot be reversed by the epigenetic modulators.

To confirm whether the alteration in expression phenotype in negative sorted populations of metastable clones upon treatment with these drugs is reflected by changes in the histone marks on the CMV promoter, we performed ChIP assays. The incubated negative sorted populations of clones 31^T and 42^T treated with NaB showed the enrichment of histone H3 acetylation marking and the reduction in the H3-K27 trimethylation levels (data not shown).

3. Discussion

In this study, we investigated the epigenetic modulation contributing to the intraclonal heterogeneity of the phenotype in the human tumor cell line HEK293T. We included GFP as a neutral reporter and isolated cells by FACS sorting, thereby avoiding any bias caused by selection pressure. In several cell clones, we observed pronounced cell-to-cell variation of the phenotype as measured by a considerable variance in GFP expression within genetically identical clonal populations of the human cell line.

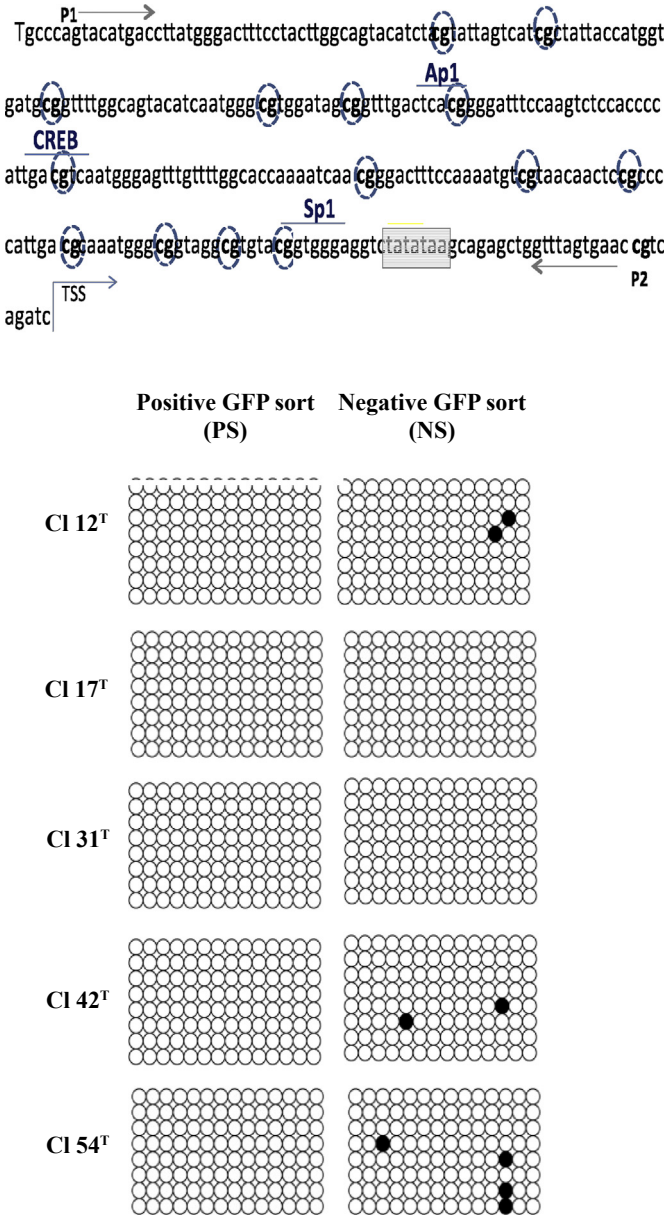

Fig. 3. Absence of DNA methylation in selected clones. The sequence of the CMV promoter region used for methylation analysis is displayed (upper part). PCR primer binding sites are indicated by arrows (P1 and P2). The transcription factor and their binding sites are labeled and 14 CpG motifs are shown by dotted circle. The TATA box is highlighted in gray. Genomic DNA was isolated from the sorted PS and NS populations of HEK293T clones and subjected to bisulfite conversion. The promoter region was amplified by PCR and integrated into a cloning vector. Random clones were picked and sequenced. The figure shows the sequences from randomly selected clones reflecting independent single cells (bottom part). Each circle in a line represents a CpG dinucleotide according to the sequence above. Unfilled circles represent non-methylated CpG dinucleotides and black filled circles represent methylated CpG dinucleotides.

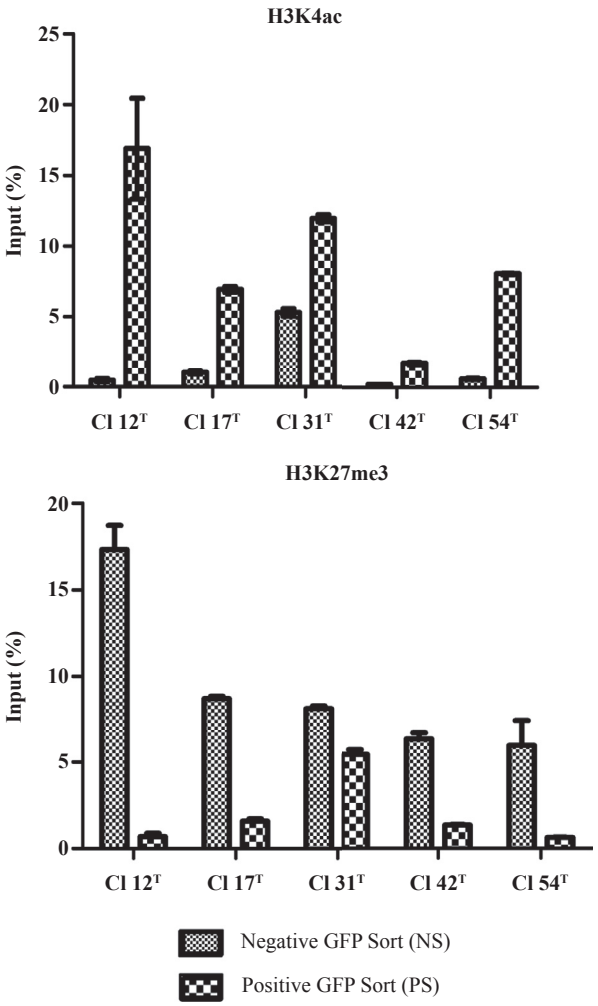

Fig. 4. Differential histone modifications in GFP expressing and non-expressing subpopulations. ChIP was performed for the sorted PS and NS fractions from the five HEK293T clones at passage 3 post sorting using antibodies against H3K4ac and H3K27me3 based on a highly standardized protocol as detailed in the Materials and Methods section. Error bars show SEM from three separate ChIP experiments. Statistical significance of the histone modifications in the NS and PS populations was confirmed by Paired *t*-test analysis for the five clones, with $P \leq 0.05$ for H3K4ac and $P \leq 0.05$ for H3K27me3.

To identify the epigenetic mechanism underlying this heterogeneity, we evaluated the impact of DNA methylation and histone modifications. We restricted our study on clones with single-copy integration events that should reflect human endogenous genes. Thereby, we could attribute the effects to a specific integration site and could exclude overlapping effects that would arise from multi-copy integrations. Thus, we excluded 'artificial cassette induced silencing' which was previously reported to accompany tandem or multi-copy integrations upon genetic modification (Eszterhas et al., 2002; McBurney et al., 2002; Ellis, 2005).

In this study, we monitored gene expression upon integrating a GFP reporter gene in a non-native surrounding. This mimics the gene translocation upon chromosomal rearrangement which is a hallmark of tumor cells. Such rearrangements can lead to disruption of evolutionarily developed chromatin states conferred by various epigenetic markings and can bring about alteration in chromatin memory. Such non-native associations result in alterations of the phenotype by overproduction or silencing of endogenous genes and even disease progression. Importantly, unlike the genetic changes, these epigenetic changes can be reversible and may account for heterogeneous phenotypes encountered within tumor populations, a fact that tumor heterogeneity severely hinders cancer therapy and also accounts for drug resistance (Feinberg et al., 2006; Knoechel et al., 2014).

We identified cell clones with large intraclonal phenotypic heterogeneity as monitored by variations in transgene expression. To study the underlying mechanism, we isolated subpopulations displaying high and low/no GFP expression, respectively, from these heterogeneous clonal populations. In the sorted

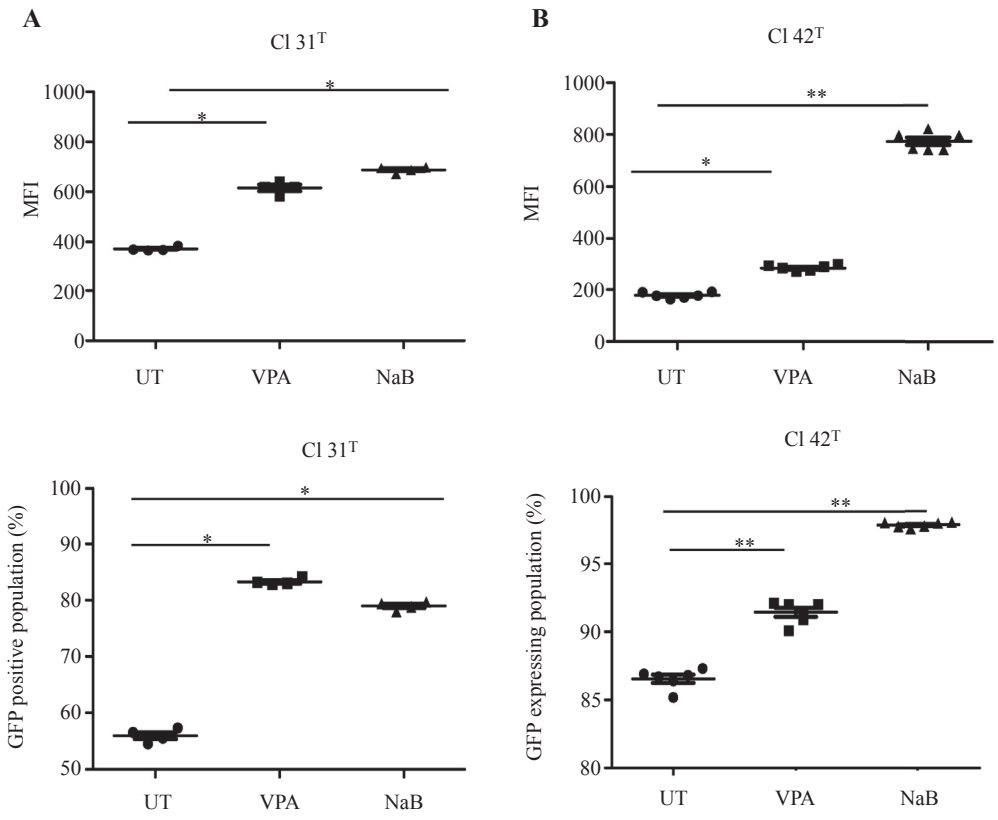

Fig. 5. Effects of HDACi on metastable clones. GFP negative cell populations (NS) from metastable clones 31^T and 42^T were treated with histone deacetylation inhibitors Valproic acid (VPA) and sodium butyrate (NaB), respectively, and subsequently analyzed for expression. Mean fluorescence intensity (MFI) as well as percentage of GFP expressing cells is shown for clone 31^T (**A**) and 42^T (**B**). The analysis was based on four and six independent samples for clone 31^T and clone 42^T, respectively. UT indicates untreated controls. *, $P \leq 0.05$; **, $P \leq 0.01$ as calculated by Mann–Whitney *U* test.

subpopulations of clones 12^T, 17^T and 54^T, the phenotype was inherited and stably maintained over passaging. In contrast, clone 31^T and 42^T showed a dynamic (metastable) phenotype with population shifting between the expressing and non-expressing states (Fig. 2B). Interestingly, in these clones, the expressing and non-expressing subpopulations merged upon cultivation. Importantly, even after repeated resorting and culturing, clones 31^T and 42^T did not give rise to a stable phenotype as these subpopulations proceeded with oscillating between expressing and non-expressing states (data not shown). Thus, these cell clones display a metastable phenotype with continuous stochastic alterations in gene expression. The shift in the expression might exemplify the plasticity observed in tumor populations. Since the transgenes of other cell clones do not exhibit such kind of metastable behavior, we conclude that the surrounding chromatin defines the epigenetic stability of the gene. As expected from the instability of the states in clones 31^T and 42^T during cultivation, these cells were highly susceptible to treatment with histone modifiers.

Importantly, we could demonstrate that the phenotype of clonal populations correlates with a specific histone modification pattern. Histone modifications have been considered to convey dynamic changes that can be triggered by slight change in the environment (Smith and Workman, 2012; Weiner et al., 2012). Upon integration of the reporter gene into the chromosome, *de novo* establishment of epigenetic marks were observed. The cell populations that showed decrease in histone H3 acetylation did not show expression of fluorescent tag GFP. The decrease of this modification has been described to result in DNA compaction since these acetyl groups neutralize the positive charges of histones and thus prevent strong interaction between histone and negatively charged phosphate group of DNA (Garcia-Ramirez et al., 1995). Also, markings like H3K27me3 have been known to lend a stably silenced phenotype in a DNA methylation independent manner. In agreement with this, a pronounced enrichment of the H3K27me3 in all the negative sorted populations was observed. However, H3K4ac marks were more frequent in all the positive sorted populations (Fig. 3). Surprisingly, DNA methylation was absent in the clonal populations that lost GFP expression in HEK293T derived clonal populations (Fig. 2B), suggesting that stable silencing is mediated predominantly by histone modifications. Importantly, complete absence of methylation was also observed even at late passages (data not shown) which signifies the stability of histone mediated repression. Further, when the cells were subjected to Decitabine, a global inhibitor of DNA methylases, no change in expression of GFP was observed (Fig. S4). Moreover, the treatment with inhibitors for DNA methylation did not result in an increase of expression in silenced cell populations (Fig. S4), suggesting that silencing of the CMV sentinel is not governed by methylation of chromosomal regions adjacent to the site of reporter integration.

Many reports have highlighted an important role of DNA methylation in particular for long term silencing of transcription (Grassi et al., 2003; Krishnan et al., 2006; Mehta et al., 2009; reviewed in Miranda and Jones, 2007). However, even in absence of DNA methylation, transcriptional repression has been observed for several endogenous genes (Lewis et al., 2004; Umlauf et al., 2004). Also, it has been demonstrated that histone based transcriptional repression precedes DNA methylation (Bachman et al., 2003; Jaenisch and Bird, 2003; Mutskov and Felsenfeld, 2004; Umlauf et al., 2004; Strunnikova et al., 2005; Miranda and Jones, 2007). Together, this suggests that DNA methylation is not

necessarily required to mediate transcriptional repression. In the clones investigated in this study, transcriptional silencing was devoid of DNA methylation even upon prolonged cultivation times, indicating that this phenotype is robust. The specific architecture of the chromosomal integration site and/or crosstalk with the integrated promoter might contribute to the stable manifestation of the silenced state in absence of DNA methylation. A more detailed analysis would be required to elucidate the molecular parameters important in this setting.

When treating the non-GFP expressing cell populations with HDACi, clones 12^T and 17^T did not respond with an increase of the expression level (Fig. S3). Thus, it seems as if these clones are locked in an epigenetically silent state. Interestingly, this state was not associated with a specific pattern of H3K27me3 or H3K4ac marks (Fig. 4), suggesting that these modifications do not allow differentiating between these expression states. From these experiments, we further conclude the key factors critically governing this state are not sensitive to VPA or NaB. Thus, it is tempting to speculate that the regulation of the histone state in these particular genomic sites is mediated by epigenetic modifiers such as histone methylase or by HDACs that are not sensitive to these two drugs (Peters et al., 2003; Dokmanovic et al., 2007). Further investigations are required to elucidate the specific epigenetic pattern that characterizes this state.

In the current study, we observed that the heterogeneity can arise from clonal cell populations that carry a single-copy sentinel cassette in a particular chromosomal site. We observed expression heterogeneity in some, but not all clones. We presume that the heterogeneity in some of the clones is a consequence of the particular chromosomal integration site and that independent integration sites have a differential impact on expression heterogeneity. Unfortunately, currently there are no available methods to predict the interaction even if the particular site would be known. This is because the various regulatory, functional and structural properties encoded by DNA elements are complex and can barely be deduced from the DNA sequence. Thus, so far, it is not feasible to theoretically predict the functional properties of a particular chromosomal site. Further, the interaction of chromatin areas is not limited to immediately adjacent chromosomal regions. An increasing number of reports highlight this complex higher-order intrachromosomal and even interchromosomal crosstalk which accounts for modulation of gene expression (Williams et al., 2010; Falvo et al., 2013).

Cell-to-cell heterogeneity can result from the consequence of transcriptional bursting. This phenomenon is based on the observation that many mammalian genes/promoters are not continuously active but rather are episodically transcribed, overall resulting in large fluctuation in individual protein levels in a single cell (Dar et al., 2012; Coulon et al., 2013). The burst time can differ with respect to the chromosomal integration site (Dar et al., 2012), usually spanning a time period of hours in mammalian cells (Raj et al., 2006). To exclude transient transcriptional bursting as an underlying mechanism of cell-to-cell heterogeneity, we based our study on a reporter with a long half-life time of more than 24 h (Corish and Tyler-Smith, 1999) which does not allow monitoring of transcriptional bursting (Dar et al., 2012). Also, we compared expression of the reporter after establishing a steady state. Thus, the observed metastability in the phenotype reflects the comparably 'slow' chromatin remodeling which is documented by the distinct histone modification pattern.

Together, the clonal populations identified here represent an example for cellular heterogeneity arising from genetically identical cells. Histone modifications were shown to be the underlying mechanism forming distinct and stable phenotypes. It would be interesting to speculate how the incoming elements would influence the homogeneity of expression of neighboring genes. Single-cell RNA sequencing might help to reveal this crosstalk.

4. Materials and methods

4.1. Mammalian cell culture and vectors

HEK293T cells (ATCC CRL-11268) were cultivated at 37°C in a humidified atmosphere with 5% CO_2. These cells were derived from HEK293 cells upon stable integration of SV40 T antigen expression vector. The human cell line was maintained in DMEM (GIBCO, USA). Culture media were supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, penicillin (10 U/mL) and streptomycin sulfate (100 μg/mL).

In the lentiviral self-inactivating tagging vector pTAG-CMV-GFP, the human CMV promoter drives *eGFP*. It was generated by ligating the *Cla* I-*Nhe* I backbone of pJSARGFP (May et al., 2008) (comprising LTRs, REV responsive element and Woodchuck hepatitis virus regulatory element (WPRE)) to a reporter cassette encoding the *eGFP* reporter gene controlled by the CMV promoter.

4.2. Lentiviral gene transfer

HEK293T cells were used for lentivirus production as specified by 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). After 12 h, the medium was replaced. After 48 h of transfection, the supernatant containing the lentiviral particles was harvested and filtered through a 45-μm filter. The virus supernatant was titered by infecting HEK293T cells with serial dilutions. For generation of single-copy tagged clones, 1×10^5 HEK293T cells were seeded and infected with 1000 viruses (MOI 0.01) in the presence of 8 μg/mL protamine sulfate. Theoretically, this gives rise to 99% of single-copy integrations from total infections. We employed a highly standardized infection protocol that was previously validated by Southern blot analysis. We routinely observed single-copy integrations in at least 95% of clones (data not shown) which is in line with the theoretical calculation (Schucht et al., 2006; Botezatu et al., 2012).

4.3. Chemical treatment

For the chemical treatment, 1×10^5 cells were seeded in a 24-well plate format. HDACi NaB (Sigma–Aldrich, USA) and VPA (Sigma Aldrich) were dissolved in PBS and added at a final molecular concentration of 1 μmol/L and 300 mmol/L, respectively. Treated and untreated control cells were cultivated for 72 h and then harvested for further analysis.

4.4. Flow cytometry

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

4.5. Chromatin immunoprecipitation (ChIP)

ChIP was done using the ChIP-IT High Sensitivity Kit (Active Motif, USA) according to the manufacturer's instructions using specific antibodies against H3K27me3 (Millipore, Cat. No. 07-449, USA) and H3K4ac (Millipore, Cat. No. 07-539). As positive and

negative controls for H3K27me3, validated primers for *MYT1* (Cat. No. 71007, Active Motif) and the human *ACTB* gene (Human Negative Control Primer Set 3, Cat. No. 71023, Active Motif) were used, respectively. As positive and negative controls for H3K4 acetylation, primers for human *ACTB-2* (Cat. No. 71005, Active Motif) and *MYT1* were used, respectively. Further, samples without antibodies were included as background controls.

For quantitative PCR, the following primers were used for the CMV promoter region: forward 5′-AAGTACGCCCCCTATTGACG-3′ and reverse 5′-AAACCGCTATCCACGCCCAT-3′. PCR reaction included the followings: 10 μL SYBR green RT-PCR mix (Qiagen, USA), 1 μL (10 mmol/L) forward primer, 1 μL (10 mmol/L) reverse primer, 8 μL immunoprecipitated DNA. Real-time PCR was performed on a LightCycler 480 apparatus (Roche, Switzerland). The reagents, primers and samples were added in a 96-well plate (Roche). All assays were performed in triplicate. The reaction was performed according to the following conditions: 95°C for 15 min followed by 45 cycles of 95°C (15 s), 58°C (20 s) and 72°C (30 s).

4.6. Bisulfite sequencing

Bisulfite sequencing was performed using the EZ DNA methylation kit (Zymo, USA) according to the manufacturer's instructions. The CMV promoter was amplified from the converted DNA using the primer pair: 5′-GTATATGATTTTATGGGATTTTTTTATTTG-3′ and 5′-ATTCACTAAACCAACTCTACTTATATAAAC-3′. The following PCR conditions were employed: 95°C for 15 min followed by 45 cycles of 95°C (30 s), 55°C (60 s) and 72°C (60 s), followed by final extension at 72°C for 7 min. Amplified PCR products were cloned into the PCR blunt cloning vector (Invitrogen, USA) using the protocol according to the manufacturer's instructions. Upon transformation and plating, eight clones were picked and expanded. Miniprep kit (Qiagen) was used to isolate plasmid DNA. As a positive control, a CMV based synthetic promoter was used that showed above 90% CpG methylation (data not shown).

Acknowledgments

This work was supported by the Initiating and Networking Fund (IVF) of the Helmholtz Association within the Helmholtz Initiative on Synthetic Biology (No. SO-078) and the Federal Ministry of Education and Research (BMBF) for the EBio project CellSys FKZ (No. 031 6189 B). Financial support was also obtained from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) (Cluster of Excellence REBIRTH as well as the project No. WI2648/3-1). We thank Maria Höxter and Lothar Gröbe for excellent support in FACS sorting. Finally, S. Spencer wishes to express his thanks for the support by the HZI GradSchool.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2016.05.008.

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