Analysis of different modes of TAL effector-mediated transcriptional regulation

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Jeannette Werner

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Promotionsausschuss:

Vorsitzender: Prof. Dr. Juri Rappsilber

Gutachter/innen: 1. Prof. Dr. Roland Lauster

2. Dr. Manfred Gossen

3. Prof. Dr. Vera Meyer

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1 Introduction

1.1 Transcriptional control of endogenous genes in eukaryotes

Eukaryotic cells evolved a complex system of gene regulation, which enables them to respond to environmental stimuli or developmental requirements. One major part of the regulation of gene expression is the control of transcription initiation as the first level for intervention. The regulatory unit of a typical eukaryotic gene consists of a promoter and *cis*-acting distal regulatory elements, which can be located several kilo bases upstream of the promoter [1].

Transcription starts with the formation of the pre-initiation complex (PIC) at the core promoter. The PIC consists of a cluster of general transcription factors and exerts diverse functions e.g. unwinding the DNA, directing RNA polymerase II (RNAPII) to the transcriptional start site (TSS) and modifying the RNAPII to release it from the promoter and enable transcription elongation [2]. Transcription, driven by these basic components on naked DNA *in vitro,* is referred to as basal transcription [1-3]. However, *in vivo* DNA is organized in nucleosomes and only the orchestration of numerous local and distal transcription factors allows high and gene specific regulation and responsiveness to environmental cues. Several *cis*-regulatory elements like proximal promoters, insulators, enhancers and silencers enable fine-tuned regulation by transcriptional activators and repressors [1].

1.1.1 Transcriptional activation

The organization of DNA in nucleosomes is overall repressive and needs to be overcome by positive regulatory elements [4]. Enhancers are regions that contain clusters of binding sites for numerous transcriptional activators, upregulating gene expression. They function largely independent on distance and orientation relative to the core promoter [1]. However, transcriptional activators often also bind in the proximal promoter or at intragenic regions. Like most transcription factors, (TF) transcriptional activators are modular and consist of a DNA-binding domain (DBD), a nuclear localization signal (NLS), an effector domain and interaction sites for cooperation with other proteins [5,6]. There are several main motifs of DBDs in mammalian transcription factors namely the helix-turn-helix, zinc finger, leucin zipper and helix-loop-helix motif [7]. Activation domains (ADs) cannot be classified that

easily, but according to their amino acid composition they can be differentiated in three categories: acidic, glutamine-rich or proline-rich [8]. Acidic domains belong to the strongest activation domains. It was shown that it is not the specific amino acid sequence but the overall negative charge that is responsible for their activation capacity [9]. One example for an acidic AD is VP16. It is derived from the herpes simplex virus type 1 and a frequently used building block for heterologous transcription factors [8].

The main mode of transcriptional activation is the regulated recruitment of binding partners near a promoter, thereby increasing the local concentration and enhancing transcription. This is often achieved with the help of the mediator complex and coactivators mediating the contact between sequence-specific factors and the general transcription machinery [10,11]. Chromatin remodeling factors, increasing DNA accessibility, are also among the recruited factors [2]. VP16 for example is known to interact with components of the PIC facilitating its assembly and recruiting histone acetyltransferases [8]. Usually many different factors bind in close proximity, resulting in a combinatorial action, which is most often synergistic rather than additive [10,12]. As a result the relative small number of about 1850 different transcription factors can regulate the expression of all genes [1]. Different combinations of sequence-specific and other regulatory elements make up a vast number of possible complexes, all acting in a differential manner. Thereby, specificity is assured by the combinatorial action of many factors and the large number of interactions rather than by high affinity or specificity of every single component, whose structure often depend on the interaction partners [4,13].

1.1.2 Transcriptional repression

As an opposing paradigm to enhancers, silencers are sequences downregulating gene expression. Similar to enhancer sequences, they can be located far up- or downstream of the promoter or even within an intron. They contain binding sites for transcriptional repressors with a negative impact on the transcription initiation rate. Like activators, repressors can act directly at the promoter site or over a longer range.

There are four major mechanisms of repression exerted by DNA-binding repressors (reviewed in [3,14]). One is the direct competition between repressors and other transcription factors for identical or overlapping binding sites whereby e.g. transcriptional activators are prevented from binding to the DNA and recruiting coactivators (see Figure 1.1 A). A second mechanism is the inhibition of the transcription machinery. Transcription initiation can be abrogated by impeding the formation of the PIC (see Figure 1.1 B). Additionally, a repressor binding in the transcribed region of a gene can obstruct transription elongation. Both, initiation and elongation can be modulated by changes in the extent or timing of covalent modifications of the RNAPII complex.

Apart from disturbing DNA-binding of TFs, repressors can interfere with transcriptional activator function as a third mechanism of repression. Preventing interactions with other TFs or masking and modifying activation domains can render activators inactive (see Figure 1.1 C). Enhancing activator degradation is an additional way to indirectly reduce transcription.

The fourth and least dynamic mechanism of repression is the remodeling of chromatin and the increase of DNA methylation (see Figure 1.1 D). Repressive heterochromatin is established through histone modifications and methylation of cytosines in a CpG context. The order of events has not been fully clarified, however it is known that heterochromatin can spread and inactivate adjacent gene loci, a process referred to as gene silencing. DNA methylation is strongly associated with reduced gene expression. About 60% of promoters are located near a CpG island, a region of 500 to 2000 bases with an elevated CG content and an accumulation of CpG dinucleotides [1]. The majority of CpGs in active promoter proximal regions is unmethlyated in contrast to single CpGs spread in the genome [1]. Increasing the methylation at promoters causes long-term silencing e.g. by blocking TF binding [15].

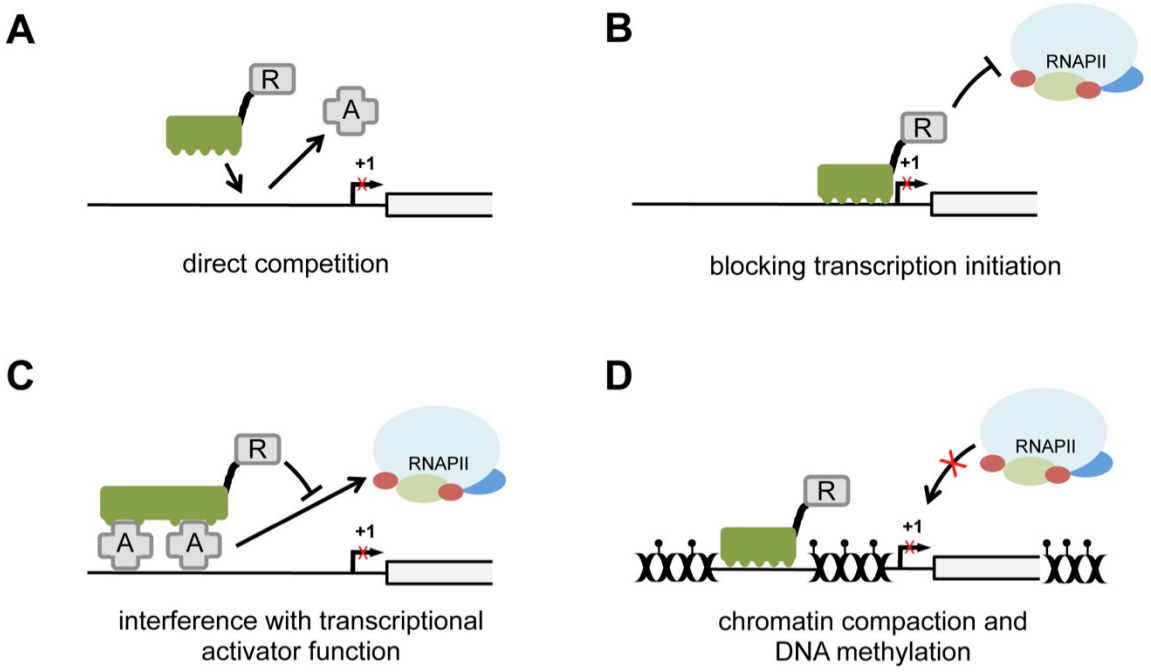

Figure 1.1 Schematic outline of the four basic repression mechanisms of DNA-bound repressors
(A) Transcriptional repressors can compete with other TFs for identical or overlapping binding sites
(B) Repressors can prevent transcription initiation by interfering with the transcription machinery.
(C) Repressors can mask the functional domains of activators and disturb their function
(D) Repressors can cause heterochromatin formation and an increase in DNA methylation, thus promoting long-term gene silencing. Activator (A); Repressor (R)
Adapted from Arnosti 2004 [14]

Whereas the first three mechanisms involve direct or corepressor-mediated contact with the transcription machinery or transcriptional activators, the remodeling of chromatin requires the recruitment of cofactors, such as histone modifying enzymes or methyltransferases. One of the better analyzed repression domains employing that mechanism is the Kruppel-Associated Box (KRAB) silencing domain [16].

1.1.3 The KRAB silencing domain

In humans, zinc finger proteins with a KRAB repression domain constitute one of the largest families of transcriptional regulators. KRAB consists of a A and B Box and acts by protein-protein interactions with corepressors and chromatin-remodeling factors (reviewed in [16]). One of them is the mandatory corepressor KRAB associated protein 1 (KAP1). KAP1 serves as a platform to coordinate the recruitment and binding of additional factors related to gene silencing, like the heterochromatin protein 1 (HP1) and histone methyltransferases [17,18]. As a result,

KRAB-mediated repression is accompanied by localized chromatin compaction and an increase in DNA methylation, making it mitotically heritable [17]. Regions with high transcriptional activity and pre-existing repressive histone-marks are especially prone to KRAB/Kap1-mediated silencing [19]. In the context of heterologous transcription factors, KRAB was shown to act as an efficient repressor when fused to the GAL4 or tetR DNA-binding domain [20,21]. Nevertheless, the long-term effect of repression from artificial factors is a matter of ongoing debate.

The transition from activation and repression is sometimes fluent. Activators can act as repressors and vice versa by a change of cofactor interaction [16,22]. The purview of both activation and repression is limited by so-called insulators that shield genes from the activities of proximal regulatory elements. Enhancer action as well as heterochromatin spreading is blocked [1].
All the above-mentioned elements allow for the gene- and context-specific transcriptional regulation that is necessary to execute complex genetic programs in a time and location dependent manner.

1.2 Artificial transcriptional control systems

The ability to regulate gene expression at will allows the analysis of gene functions and interactions and has a large field of application. Several systems acting at the level of transcription initiation have been established, of which three are shortly introduced below.
In 1961, Jacob and Monod established the concept of gene regulation on the basis of the *lac* operon in *E. coli* [23]. The expression of genes needed to metabolize lactose is negatively regulated by the binding of the *lac* repressor to a *cis*-regulatory element of the *lac* operon in the absence of the metabolite. In the presence of lactose, this repression is abrogated and the cell is able to utilize lactose as an energy source. From its first description, the *lac* system has been refined and widely employed in different organisms [24-26].
The GAL4/UAS system has been especially useful for tissue-specific gene expression in *Drosophila* [27]. It is based on the yeast transcription factor GAL4, which can be placed under the control of a tissue-specific endogenous promoter. The GAL4 binding site, namely the UAS (Upstream Activating Sequence), is engineered upstream of a reporter gene or a gene of interest. Tissue-specific or developmentally

controlled expression of GAL4 results in the induction of the UAS controlled gene [27]. The GAL4 transcriptional activator and the UAS controlled gene are often split in so-called driver and responder lines and target gene expression is only induced upon mating. This enables the expression of toxic or lethal genes [28]

The by far most widely applied transcriptional control system in eukaryotes is the Tet system. It originates from *E. coli* where it regulates the resistance to tetracycline. The tet repressor (tetR) protein binds as a dimer to the 19 bp tet operator (*tet*O) in the Tn10 tetracycline resistance operon and inhibits expression of the resistance protein tetA. If the cell encounters tetracycline in its environment, the binding of tetR is reversed and transcription of the resistance gene is initiated [29]. In 1992, Gossen and Bujard established the Tet system for transcriptional control in mammalian cells [30]. Since then, the Tet system has developed into the best characterized and most widely applied gene control system. There are two basic variants. The initially developed Tet-Off system uses tTAs, a tet repressor fused to a transcriptional activation domain, and a minimal promoter containing multimerized *tet*O sequences as tetR binding sites [30]. In the absence of tetracyclines or derivates like doxycycline (dox), tTAs is bound to *tet*O and transcription is initiated. In the presence of dox this binding is prevented resulting in deactivation of transcription (see Figure 1.2 right panel). The subsequently established Tet-On system relies on rtTA, a variant of tTA, which depends on dox for binding to *tet*O sequences and inducing transcription [31] (see Figure 1.2 left panel).

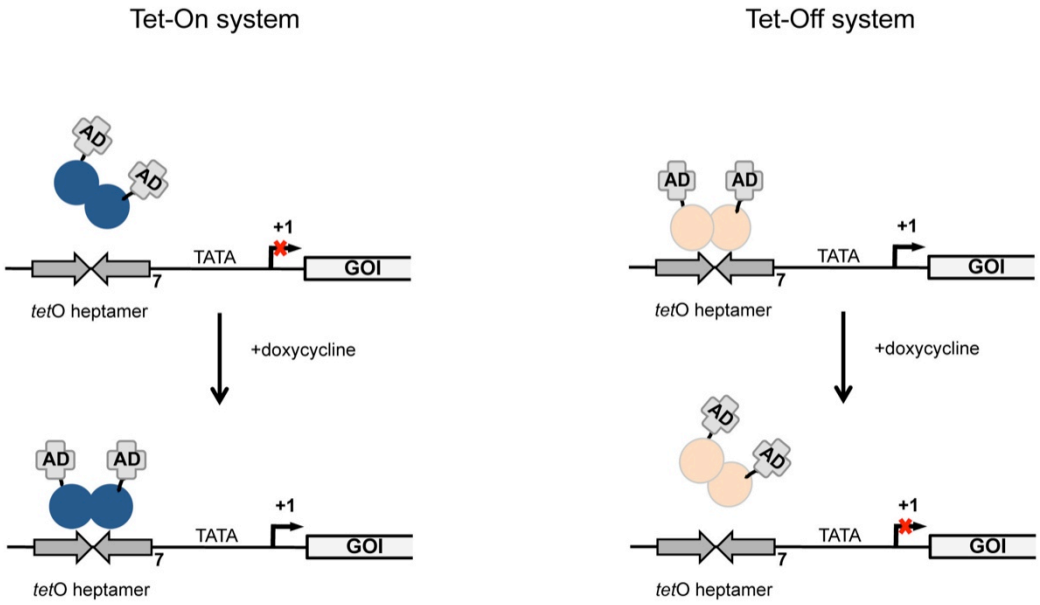

Figure 1.2 Schematic outline of the basic principle of the Tet-On and the Tet-Off system
The Tet-On system uses rtTA, a fusion of a modified tet repressor and an activation domain (AD). rtTA depends on doxycycline for binding to the tet operator (left panel). Upon binding it induces the transcription of a gene of interest (GOI).
The Tet-Off system relies on the original tTAs, also containing an AD. tTAs can only bind and induce transcription in the absence of doxycycline (right panel).

Components of both systems have undergone optimization with respect to background expression and inducibility [32,33]. They are widely applied in basic science, both in cell models [34,35] and transgenic organisms [36], but also in the production of recombinant proteins [37] and synthetic biology [38].

The advancement in the field of artificial transcriptional control systems over the last five decades has considerably contributed to basic science, biotechnological production, gene therapy and the production of transgenic animals. Despite the advantage of fast kinetics and a high regulatory window, all systems described so far share the limitation that cells need to be genetically engineered to carry effectors as well as the target sequences. This makes them difficult to employ for the control of endogenous genes. The regulation of endogenous genes requires the development of efficient and specific targeting moieties adaptable of binding almost any DNA sequence at will. Only this will bring the application of transcriptional control system as efficient therapeutic agents within reach.

1.3 Designer transcription factors

Several such DNA sequence-specific binding agents have been reported over the last decades. Triplex forming oligonucleotides for example can bind to the major groove of DNA in a sequence-specific manner. However, despite optimization efforts, they are largely restricted to purine-rich targets and were shown to increase mutagenesis [39]. Furthermore, synthetic polyamides have been used to target endogenous genes. They bind to the minor groove of DNA and pairs of amino acids specifically form hydrogen bonds with individual DNA bases. Those polyamides have good cell permeable properties but are restricted to relatively short target sequences [39].

The most frequently used tools for tailor-made DNA-binding domains are zinc finger proteins (ZFP) and more recently transcription activator-like effectors (TALE) as well as clustered regularly interspaced short palindromic repeat (CRISPR)-based complexes. All of these designer DBD have been developed and characterized as fusions, rendering them capable of acting as designer nucleases in the context of genome editing applications. In the following, they are mainly introduced in their capability as heterologous transcription factors.

1.3.1 Zinc finger proteins

Zinc fingers are the most abundant DNA-binding motifs in eukaryotes [39]. The canonical zinc finger motif is C2H2: two cysteines in the N-terminal β-sheets and two histidine residues in the C-terminal α-helix interact with a stabilizing zinc atom. Specific DNA interaction is mediated by a short amino acid stretch in the α-helix through interaction with one DNA strand [16,39]. Each finger recognizes three to four partially overlapping bases. As tools in transcriptional control systems, zinc fingers have the major advantage that they bind as a monomer and do not need symmetrical target sites. However, as there is no recognition code of one amino acid to one DNA base, the design and testing of ZF proteins is elaborate. Furthermore, the binding of one zinc finger is not completely independent of the neighboring finger, further complicating the design. Still, different methods for effective screening have been established like the phage display technique and the bacterial two-hybrid system. Together with careful target site selection, particularly with regard to DNA accessibility, those methods led to the successful use of ZFP for the regulation of

endogenous genes (reviewed in [39]). Their specificity was demonstrated by the unaltered expression of target gene family members [40] or by performing gene array analysis [41]. They were shown to be able to activate silenced genes [42] and there is evidence that they can remodel the chromatin [43]. Further development included the establishment of a cell permeable zinc finger [44] and the fusion of a histone methyltransferase for long-term repression [45]. For the design of new zinc finger transcription factors, natural equivalents can be used as a scaffold and adjusted by modifying the amino acid composition at the critical sites. The most potent effector needs to be determined empirically as it is highly context-dependent [39]. Instead of putting the target site first, Barbas' group followed an approach where a whole library of 3-finger ZFP was transduced in cells, which were subsequently checked for target gene expression. Zinc finger domains were recovered from positive cells and subjected to another round of selection. This resulted in the identification of ZFs specifically enhancing the expression of specific target genes [46]. Notwithstanding the fact that zinc finger DBDs have been successfully used for endogenous gene regulation and improved assembly methods have been established [47], the complexity of design and functional screening prevented it from becoming a routine method.

1.3.2 Transcription activator-like effectors (TALEs)

Transcription activator-like effectors (TALEs) are *trans*-kingdom transcription factors from plant pathogenic bacteria like *Xanthomonas* or *Ralstonia* species [48]. The bacteria secret TALEs into the plant cell where they are transported to the nucleus and induce the transcription of genes that facilitate infection or elicit defense in case of resistant plants [49]. The N-terminal region of TALEs contains signals for translocation into the plant cell, whereas the nuclear localization signal and an acidic activation domain are located in the C-terminal part of the protein. The central region consists of tandem repeats of mostly 34 amino acids, which only differ in two amino acids at position 12 and 13, also called repeat variable diresidue (RVD) [49]. In 2009, two groups independently published the so-called TALE-code, assigning specific repeats to specific DNA bases, thereby paving the way for a new class of designer DNA-binding proteins with broad application potential [50,51]. Boch *et al*. realized that in the TALE AvrBs3 the number of repeats coincides with the number of recognized DNA bases. They established a theory of one repeat recognizing one

DNA base. By target site prediction for other known TALEs and reporter assays they could indeed show that each RVD (HD, NG, NI and NN) specifically recognize one DNA base (C, T, A and A/G, respectively). By producing artificial TALEs with newly arranged repeats, they demonstrated their modular nature and the applicability of TALEs as artificial transcription factors. Moreover, they showed that a poorly conserved region recognizing a thymine precedes the central repeats and is mandatory for TALE function. The last repeat is truncated and referred to as a half repeat [50]. The group of Moscou *et al*. largely came to the same conclusions by a computationally approach [51].

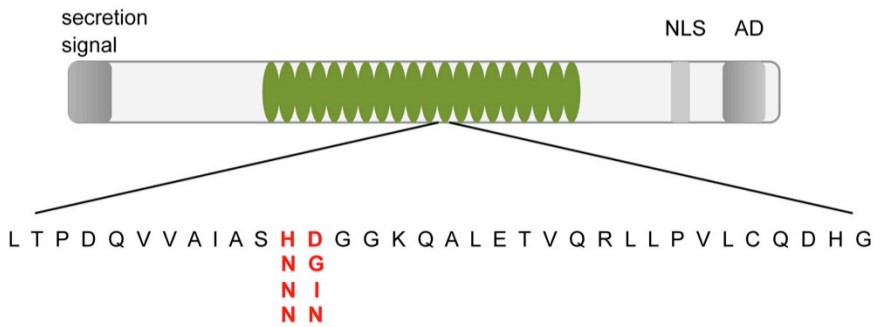

Figure 1.3 Schematic representation of natural TALE structure
The N-terminal part of the TALE protein includes the signal for secretion into the plant cell, whereas the C-terminal region comprises the signal for nuclear transport and a transcriptional activation domain. The central region is composed of tandem repeats, varying only in two amino acids (depicted in red) relevant for specific DNA recognition. Nuclear localization signal (NLS); activation domain (AD). Adapted from Boch *et al*. [50]

Most natural TALEs contain 15.5 to 19.5 repeats of 34 amino acids each [52]. A minimum of 6.5 repeats is necessary, but 10.5 or more result in an increasing transcriptional activator functionality [50]. In plants, TALE binding defines the transcriptional start site and presumably helps assemble the PIC in a way similar to the TATA-box-binding protein [49,52]. Analysis of TALE structure revealed that the RVDs of each repeat are located in a loop connecting two left-handed helices [48]. The amino acid at position 13 is responsible for the specific DNA interaction through hydrogen bonds or van der Waals interactions, whereas the amino acid at position 12 is not involved but stabilizes the TALE structure [53]. The tandem repeat array wraps around the major groove of the DNA with individual repeats interacting with only one strand of the DNA [53].

Since the initial publications on DNA recognition by TALEs, a number of construction protocols have been established making the TALE technology available to a broad

research community [54-57]. Optimizations with regard to protein truncations and alternative repeats have improved TALE functionality and specificity, especially with respect to the recognition of modified DNA bases like methylated cytosines [55,58-61]. Several guidelines concerning target site selection and repeat composition have emerged with their stringency being subject of controversial debate [54,58,60,62].
A wide range of functional domains have been fused to TALEs, including the hydroxylase TET1 [63], the histone demethylase LSD1 [64], the recombinase Gin [65] and the *piggyBac* transposase [66]. However, most efforts are concentrated on TALE nucleases for genome editing. The focus of this thesis is the use of TALEs as designer transcription factors with emphasis on possible modes of repression, a topic only sporadically touched in the literature [59,67-69].
In contrast to repression, activation of reporter and endogenous genes is the subject of intensive research and has been described in a number of publications [55,70-74]. Yet, 22 out of 26 studies do not achieve an activation of endogenous genes by more than five-fold [75]. This modest activation is commonly attributed to a lack of DNA accessibility, especially when silent loci are targeted [55,74]. This problem has in part been solved by the observation that the simultaneous use of multiple TALEs results in a synergistic activation of target genes [75,76]. However, until now no systematic study was published addressing the power of TALE-based TFs compared to that of other heterologous systems.

1.3.3 CRISPR/Cas

In short succession to TALEs, another system to sequence specifically target DNA at will has emerged, namely the bacterial CRISPR/Cas system (Clustered regularly interspaced short palindromic repeats/CRISPR associated proteins). As a tool for genome engineering, the focus is on the type II system from *Streptococcus pyogenes* due to its relative simplicity. In its natural context it serves as an adaptive defense mechanism against foreign DNA [77]. Foreign DNA is integrated as a spacer in so-called CRISPR arrays. Transcription of these regions is followed by the processing of the CRISPR RNA (crRNA) with the help of the *trans*-acting RNA (tracrRNA), RNAseIII and the endonuclease Cas9. After maturation crRNA, tracrRNA and Cas9 form an endonuclease complex that binds complementary invading DNA, flanked by a specific protospacer adjacent motif (PAM), and causes blunt double strand breaks [78]. In 2013, two groups adapted that system for the use in mammalian cells.

Instead of two separate RNAs a fusion, a so-called single guide RNA (sgRNA) transcribed from a RNAP III promoter, was used to direct the codon-optimized Cas9 nuclease to a specific target [79,80]. Since then, the use of the CRISPR/Cas system for genome engineering was demonstrated for different targets in different cell types [81-83].

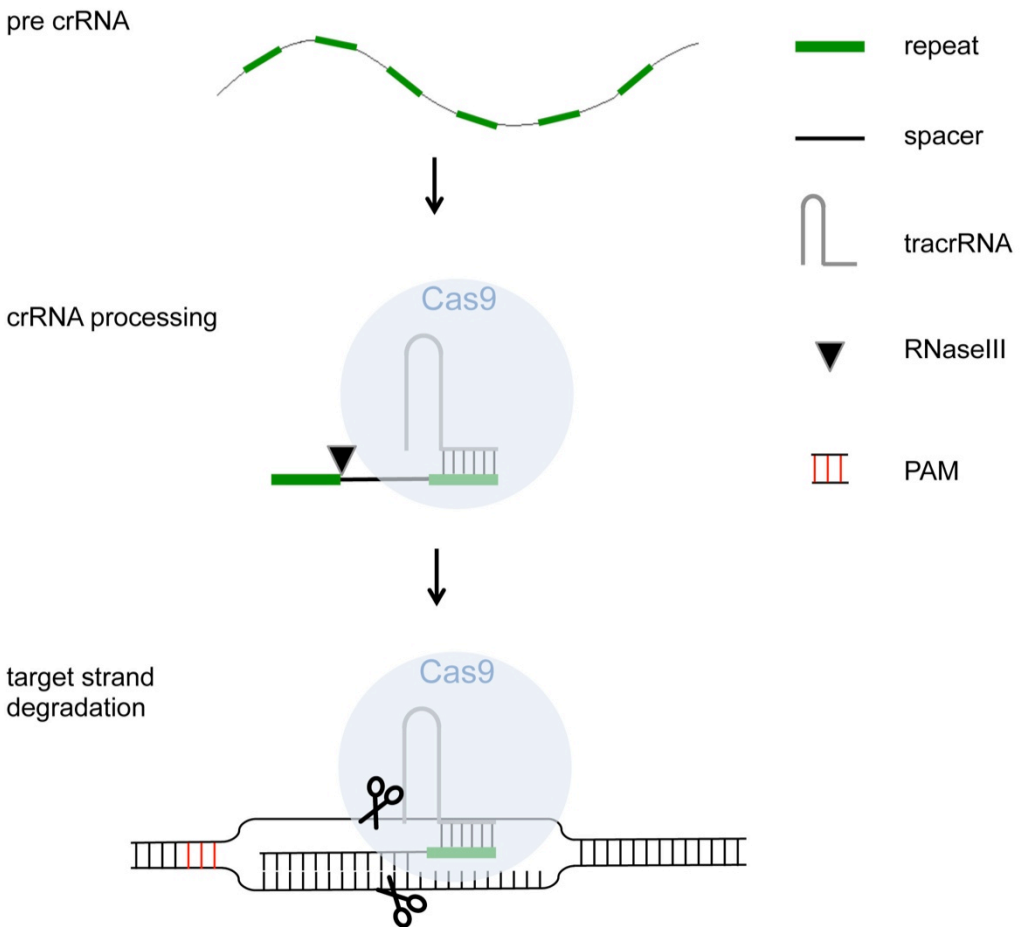

Figure 1.4 Schematic representation of crRNA maturation and CRISPR/Cas action
The transcription of spacer and repeat sequences (pre-crRNA) is followed by crRNA processing and the formation of a crRNA/tracrRNA/Cas9 complex. In *Streptococcus pyogenes* this complex targets invading sequences, complementary to the spacer region of the crRNA, and induces double strand breaks.

By introducing mutations in the Cas9, rendering it catalytically inactive, the spectrum of applications has broadened. This allows the fusion of other functional domains and thereby the use of the system's components for transcriptional control. The ability to specifically activate and repress reporter and endogenous genes was demonstrated by several studies [84-87], and as for TALEs the use of several sgRNAs at the same time was shown to result in a synergistic action [84,86,88]. Moreover, the use of sgRNAs facilitates multiplexing, i.e. the targeting of several genes at the same time

only by co-delivering multiple sgRNAs. Despite the fact that in principle every sequence can be targeted as long as it is flanked by the PAM sequence, several groups report unpredictable malfunction of sgRNAs [87,89] and in some cases lower activation capacity as compared to TALEs targeting the same genes [84,86].

Taken together, the CRISPR/Cas system constitutes a useful tool to direct a multitude of functional domains to specific DNA sequences with some issues leaving room for improvement.

1.4 Aim of the study

The directed modulation of endogenous gene expression is the key for elucidating interdependencies of genes in basic research and the therapeutic intervention in diseases resulting from transcriptional misregulation.

In this thesis the focus was on transcription activator-like effectors (TALEs). These tailored DNA-binding proteins are a versatile tool to target largely any genomic locus. The fusion with transcriptional regulatory domains makes them applicable as designer transcription factors. Despite the numerous advantages, many studies using TALEs encounter the problem of no or insufficient performance for reasons not yet known. However, for the routine application such failures need to be eliminated. To this end, a deeper understanding of the basic mechanism of efficient gene regulation with this kind of designer transcription factors is required. This study sought to systematically evaluate TALE-based transcription factors and provide indications on how the potential of the TALE technology can be fully exploited. This new generation of designer transcription factors can considerably benefit from the knowledge gained through intense research on “classical” artificial control systems adapted for transcriptional control in mammalian cells. Therefore, a comparison of TALE transcription factors to the well established Tet system was made, targeting the by far best characterized binding site for promoter activation, namely the tet operator. This allows an assessment of the maximum efficacy that can be achieved.

Efficient repression is a field only marginally examined so far although it is an indispensable aspect for situations where the limitation of the expression of a set of genes is decisive for the desired outcome. Consequently, special focus was placed on the mechanistic investigation on TALE-mediated repression, including *trans*-repression, competition with other DNA-binding factors and blocking of transcription elongation.

Furthermore, to combine the best traits of both systems and enable further investigation concerning reversibility, the study aimed at establishing an inducible TALE transcription factor. This enables unprecedented flexibility in applications where a temporary intervention is required.

As the CRISPR/Cas system emerged in the course of this thesis as an alternative, it was partly included in the comparative part of the study to gain first insights in terms of efficiency of these evolutionary distant control systems, targeting an identical binding site.

2 Materials

2.1 Chemicals

The chemicals used in this thesis are listed in Table 1.

Table 1. Chemicals

Name	Company	Storage
Agarose	Invitrogen	RT
Agar	Difco/BD	RT
Ampicillin	Roth	4°C
ATP	AppliChem	-20°C
Blasticidin	Invivogen	-20°C
Blocking buffer	LiCor	4°C
Bovine serum albumin	AppliChem	4°C
Bromphenol blue	AppliChem	RT
Coelenterazine	Biosynth	-20°C
DMEM	Sigma-Aldrich	4°C
DMSO	Roth	RT
dNTPs	Invitrogen	-20°C
Dox	Sigma-Aldrich	4°C
EDTA	Roth	RT
Ethanol	Roth	RT
Ethidium bromide	AppliChem	RT
Fetal calf serum	Biochrom	-20°C
Glycerol	Roth	RT
A/C Heterodimerizer	Clontech	-20°C
IPTG	Zymo Research	-20°C
L-Glutamine	Sigma-Aldrich	-20°C
LB medium	Roth	RT
Lenti-X Concentrator	Clontech	4°C
LSM 1077 Lymphocyte	PAA	RT
Luciferin	Sigma-Aldrich	-20°C
Non-essential amino acids	Gibco	-20°C
NP-40	Sigma-Aldrich	RT

Table 1 (continued)

Name	Company	Storage
NuPAGE MOPS	Life Technologies	RT
Odyssey blocking buffer	Licor	4°C
PCR reagents	Invitrogen	-20°C
Penicillin/Streptomycin	Sigma-Aldrich	-20°C
Phosphate Buffered Saline	Gibco	4°C
Polyethylenimine (linear)	Polysciences	-20°C
Ponasteron A	Invitrogen	-20°C
Puromycin	Invivogen	-20°C
RPMI	Gibco	4°C
Salmon sperm DNA	Sigma-Aldrich	-20°C
Sodium chloride	Roth	RT
Sodium pyruvate	Gibco	-20°C
Spectinomycin	AppliChem	4°C
Tetracycline	Sigma-Aldrich	4°C
Tris base	Sigma-Aldrich	RT
Trypane blue	Gibco	RT
TrypLE Select	Gibco	4°C
Tween 20	Roth	RT
X-Gal	Invitrogen	-20°C
Zeocin	Invivogen	-20°C
β-mercaptoethanol	Sigma-Aldrich	RT

2.2 Consumables

The consumables used in this thesis are listed in Table 2.

Table 2. Consumables

Name	Company
BD Plastipak 1ml Sub-Q	BD
Cell culture flasks	TPP
Cell culture plates	TPP
Electroporation cuvettes	BioRad
Eppendorf tube	Eppendorf

Table 2 (continued)

Name	Company
Falcon polystyrene round bottom tube	BD Falcon
Nitrocellulose membrane	Millipore
NuPage Bis-Tris precast gels 4-12%	Life Technologies
NuPAGE Novex 4-12% Bis-Tris gradient gel	Life technologies
PCR tubes	Eppendorf
Serological pipette	BD Falcon
Whatman blotting paper	Sigma-Aldrich

2.3 Enzymes

The enzymes used in this thesis are listed in Table 3.

Table 3. Enzymes

Name	Company
Taq DNA polymerase	Invitrogen
T4 Quick DNA Ligase	New England BioLabs Inc.
T4 Polynucleotide kinase	New England BioLabs Inc.
SuperSAP	Affymetrix
PWO polymerase	Roche
HotStarTaq polymerase	QIAGEN
All restriction enzymes	New England BioLabs Inc.

2.4 Antibodies

The antibodies for immunoblot analysis used in this thesis are listed in Table 4.

Table 4. Antibodies

Name	Host	Company	Storage
anti-HA	rabbit	Sigma-Aldrich	-20 °C
anti-β-actin	mouse	Sigma-Aldrich	-20 °C
IRDye 800 CW donkey anti-rabbit	donkey	Licor	-20 °C
IRDye 680 donkey anti-mouse	donkey	Licor	-20 °C
anti-EGFP	rabbit	-	-20 °C

2.5 Buffers and solutions

Cell culture medium, buffers and solutions used in this thesis are listed in Table 5.

Table 5. Buffers and solutions

Buffer	Composition
HAFTL culture medium	10% heat inactivated FCS 100 units/ml Pen/Strep
HeLa culture medium	DMEM 10% FCS 2 mM glutamine 100 units/ml Pen/Strep
HEK293 culture medium	DMEM 10% FCS 4 mM glutamine 10 mM non-essential amino acids 1 mM sodium pyruvate 100 units/ml Pen/Strep
CHO K1 culture medium	RPMI 1640 10% FCS 100 units/ml Pen/Strep
Jurkat culture medium	RPMI 1640 10% FCS 100 units/ml Pen/Strep
FACS buffer	PBS 2% BSA 2mM EDTA
TBS	50 mM Tris-Cl pH 7.6 150 mM NaCl
TBS-T	50 mM Tris-Cl pH 7.6 150 mM NaCl 0.05% Tween 20
SDS sample buffer	100 mM Tris-HCl pH 6.8 4% SDS 20% glycerol 2% β-mercaptoethanol 25 mM EDTA 0.04% bromophenol blue

Table 5 (continued)

Buffer	Composition
DNA loading buffer	60 mM Tris-HCl pH 7.5 60 mM EDTA 60% glycerol 1x bromophenol blue
Wet transfer buffer	25mM Tris 192 mM glycine 20% methanol
TAE buffer	10 mM Tris-HCl 1 mM EDTA
Lysis buffer	PBS 0.25% NP40
Luciferase reaction buffer	25 mM glycylglycine 15 mM $MgSO_4$
Firefly luciferase substrate	25 mM glycylglycine 15 mM $MgSO_4$ 5 mM ATP 200 µM luciferin
Renilla luciferase substrate	25 mM glycylglycine 15 mM $MgSO_4$ 4 µM coelenterazin

2.6 Machines

Machines used in this thesis are listed in Table 6.

Table 6. Machines

Name	Company
Accuri C6 Flow cytometer	BD Bioscience
Agarose gel chamber	Thermo Scientific
Axio Observer	Zeiss
Bacteria incubator	Memmert
BD FACSAria™ III	BD Bioscience
BD LSR II Flow cytometer	BD Bioscience
Bunsen burner	Integra Bioscience

Table 6 (continued)

Name	Company
Cell culture incubator	Binder
Centrifuge Avanti J-26 XP	Beckman Coulter
Eppendorf centrifuge 5417R	Eppendorf
Eppendorf centrifuge 5810R	Eppendorf
Fridge Freezer	Liebherr
Gel documentation	Berthold Technologies
Gene Pulser XCell	BioRad
Improved Neubauer Haemocytometer	Reichert
Innova 44 Incubator shaker series	New Brunswick Scientific
Laminar flow cabinet	Thermo Scientific
Microscope Leica DM-IL	Leica
Mighty Small wet blotting system	Amersham Biosciences
Mithras LB 940	Berthold Technologies
Mr. Frosty Freezing Container	Nalgene
NanoDrop 1000	Peqlab
Odyssey infrared imaging system	LiCor
Pipettes	Eppendorf
PowerPac 300	BioRad
Thermocycler comfort	Eppendorf
Vacusafe comfort	Integra Bioscience
Vortexer	VWR
Water bath	GFL

2.7 Molecular biology kits

Kits used in this thesis are listed in Table 7.

Table 7. Kits

Name	Company
EpiTect Bisulfite Kit	QIAGEN
NucleoBond Xtra	Macherey-Nagel
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel
NucleoSpin Plasmid	Macherey-Nagel

Table 7 (continued)

Name	Company
NucleoSpin Tissue	Macherey-Nagel
Pierce BCA Protein Assay kit	Thermo Scientific

2.8 Cell lines

Cell lines used in this thesis are listed in Table 8.

Table 8. Cell lines

Name	ATCC number/ Reference	Description
HAFTL	Holmes *et al*. [90]	mouse B-cell progenitor
HAFTL tetEF-ZsGreen		carry multiple *tet*O sequences in a TRE context upstream of the hEF1α promoter, driving the expression of the destabilized ZsGreen reporter (M. Hofstätter and M. Gossen, unpublished data).
Jurkat	TIB-152	human acute T cell leukemia
CHO K1	CCL-61	chinese hamster ovary
CHO Tet-On Advanced	Clontech	carry an expression cassette for the reverse tet *trans*-activator (rtTA)
HeLa	CCL-2	human cervix cancer
X1/5	Gossen *et al*. [30]	stably transfected with pUHC13-3, carrying a tet-responsive promoter upstream of a luciferase reporter as well as an expression cassette for the tet *trans*-activator tTA
X1/6	Baron *et al*. [33]	carry a tet-responsive luciferase reporter but no functional *trans*-activator gene
HEK293 TN	CRL-1573	human embryonic kidney
HEK Tet-On Advanced	Clontech	carry an expression cassette for the reverse tet *trans*-activator (rtTA)

2.9 Bacterial strains

Cell lines used in this thesis are listed in Table 9.

Table 9. Bacterial strains

Name	Genotype
Top10 F1	F´{*lac*Iq, Tn*10*(TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM1 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A *nup*G
DH5alpha	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK-mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1
Stbl3	F– *mcr*B *mrr hsd*S20 (rB–, mB–) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y *pro*A2 *rps*L20 (StrR) *xyl*-5 λ– *leu mtl*-1

2.10 Software

Cell lines used in this thesis are listed in Table 10.

Table 10: Software

Name	Company
AxioVision Rel. 4.8	Zeiss
BD Cflow Plus 1.0	BD
BD FACSDiva 8.0.1	BD
FlowJo 9.5.2	FlowJo, LLC
GraphPad Prism 5.0	GraphPad Sofware
Microsoft Office 2011	Microsoft
MikroWin 2000	Berthold
Serial cloner 2-5	Serial Basics

2.11 Constructs

Constructs and the figures they were used in in this thesis are listed in Table 11.

Table 11: Constructs

name	description	used in Figure
pMD20	TA cloning vector	
psPAX2	lentiviral packaging plasmid (gag and pol)	
pMD2.G	lentiviral packaging plasmid (VSV-G glycoprotein)	
reporter		
P_{tet1} luc	reporter carrying a tet-responsive promoter with 1 *tet*O sequence upstream of a luciferase reporter (published in [30])	Figure 4.2 B
P_{tet7} luc	reporter carrying a tet-responsive promoter with 7 *tet*O sequences upstream of a luciferase reporter (published as pUHC13-3 in [30])	Figure 4.1 B/4.2 B/4.4 A / 4.5 A/4.6 A/ 4.7 A/4.8 A/4.50
$P_{tet(rev)7}$ luc	reporter carrying a tet-responsive promoter with 7 *tet*O sequences in reverse orientation upstream of a luciferase reporter (published as pUHC13-4 in [30])	Figure 4.4 A
$P_{tet(4C)7}$ luc	reporter carrying a tet-responsive promoter with 7 *tet*O sequences comprising two mismatches upstream of a luciferase reporter (published as pUHC13-8 in [91])	Figure 4.5 A
$P_{tet(6C)7}$ luc	reporter carrying a tet-responsive promoter with 7 *tet*O sequences comprising two mismatches upstream of a luciferase reporter (published as pUHC13-9 in [91])	Figure 4.5 A
pTRE d2EGFP	reporter carrying a tet-responsive promoter with 7 *tet*O sequences upstream of a d2EGFP reporter	Figure 4.2 A

Table 11 (continued)

name	description	used in Figure
pUHC13-13	reporter carrying a constitutively active tet-responsive promoter with 7 *tet*O sequences upstream of a luciferase reporter	Figure 4.9 B/4.10 A/ 4.10 B/4.11/ 4.51
SB tetEF-ZsGreen	hEF1α driven ZsGreen reporter (*Sleeping Beauty* Transposon)	Figure 4.13/4.14/4.33/ 4.53
SB tetRosa-ZsGreen	hRosa driven ZsGreen reporter (*Sleeping Beauty* Transposon)	Figure 4.15
SB tetUbc-ZsGreen	hUbC driven ZsGreen reporter (*Sleeping Beauty* Transposon)	Figure 4.15
tetEF d2EGFP	Lentiviral hEF1α driven d2EGFP reporter	Figure 4.17
SB EF EYFPi	hEF1α driven EYFP reporter with an artificial intron (*Sleeping Beauty* Transposon)	Figure 4.19/4.20
SB EF EYFPi $tetO_1$	hEF1α driven EYFP reporter with an artificial intron containing one copy of the *tet*O (*Sleeping Beauty* Transposon)	Figure 4.19/4.20
SB EF EYFPi $tetO_7$	hEF1α driven EYFP reporter with an artificial intron containing seven copy of the *tet*O (*Sleeping Beauty* Transposon)	Figure 4.19/4.20
$P_{tet7(bi)}$ EGFP/luc	bidirectional P_{tet7} driving a luciferase and EGFP reporter	Figure 4.42/4.44
SB CMV $tetO_2$ EGFP	CMV driven EGFP reporter with two copies of the *tet*O located between the TATA box and the transcriptional start site of the CMV promoter (derived from the commercially available pCDNA4To_mcs at invitrogen) (*Sleeping Beauty* Transposon)	Figure 4.45
SB DmrC/DmrA	iDimerize activator components (*Sleeping Beauty* Transposon)	Figure 4.22
PB tTAs	hEF1α driven tet *trans*-activator (*piggyBac* Transposon)	Figure 4.1 B/4.2 A/4.2 B / 4.3/4.4 A/4.5 A/ 4.50

Table 11 (continued)

name	description	used in Figure
tetR	CMV driven tet repressor (commercially availabe as pcDNA6/TR at Invitrogen)	Figure 4.45/4.52
tetR-SD	CMV driven tet repressor fused to a KRAB domain	Figure 4.9 B/4.51
PB tetR	EF driven tet repressor (*piggyBac* Transposon)	Figure 4.20
TALE constructs		
tetTALE-AD RFP	hEF1α driven *tet*O targeting TALE fused to a VP64 activation domain and a T2A linked RFP marker	Figure 4.1 B/4.2 A/4.3/ 4.6 A/4.7 A
SB HA tetTALE-AD RFP	hEF1α driven *tet*O targeting TALE fused to a VP64 activation domain and a T2A linked RFP marker with a N-terminal HA tag (*Sleeping Beauty* Transposon)	Figure 4.7 A
SB HA tetTALE-AD mCherry	hEF1α driven *tet*O targeting TALE fused to a VP64 activation domain and a T2A linked mCherry marker with a N-terminal HA tag (*Sleeping Beauty* Transposon)	Figure 4.24/4.25
tetTALE-AD 5mm	hEF1α driven *tet*O targeting TALE carrying 5 additional C-terminal repeats not matching the *tet*O sequence fused to a VP64 activation domain	Figure 4.6 A
tetTALE-AD first T mm	hEF1α driven *tet*O targeting TALE with the first T nor matching the sequence fused to a VP64 activation domain	Figure 4.6 A
PB tetOTALEpart-AD 1 mCherry	hEF1α driven TALE partly targeting the *tet*O fused to a VP64 activation domain and a T2A linked mCherry marker (adapted from the previously described *tet*O targeting TALE in[92]) (*piggyBac* Transposon)	Figure 4.8 A

Table 11 (continued)

name	description	used in Figure
PB tetOTALEpart-AD 2 mCherry	hEF1α driven TALE partly targeting the *tet*O fused to a VP64 activation domain and a T2A linked mCherry marker (adapted from the previously described *tet*O targeting TALE in[92]) (*piggyBac* Transposon)	Figure 4.8 A
PB EF HA tetTALE-SD mCherry	hEF1α driven *tet*O targeting TALE fused to a KRAB silencing domain and a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.10 B/4.11/ 4.13/4.14 A/4.15/ 4.19/4.20/4.42/ 4.44
PB CMV HA tetTALE-SD mCherry	CMV driven *tet*O targeting TALE fused to a KRAB silencing domain and a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.9 B/4.17/4.51
PB CMV HA tetTALE mCherry	CMV driven *tet*O targeting TALE without transcriptional regulatory domain but a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.17/4.45/4.52
SB HA tetTALE-SD EGFP	hEF1α driven *tet*O targeting TALE fused to a KRAB silencing domain and a T2A linked EGFP marker with a N-terminal HA tag (*Sleeping Beauty* Transposon)	Figure 4.10 A/4.35/4.37/ 4.39
PB EF HA tetTALE mCherry	hEF1α driven *tet*O targeting TALE without transcriptional regulatory domain but a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.13/4.15/4.19/ 4.20/4.42/4.44/ 4.46/4.47/4.48
PB EF HA tetTALE$_{rev}$ mCherry	hEF1α driven TALE targeting the opposite strand of *tet*O without transcriptional regulatory domain but a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.20/4.46
SB HA tetTALE EGFP	hEF1α driven *tet*O targeting TALE without transcriptional regulatory domain but a T2A linked EGFP marker with a N-terminal HA tag (*Sleeping Beauty* Transposon)	Figure 4.10 A/4.35/4.37 A/4.39 A/4.40 A

Table 11 (continued)

name	description	used in Figure
PB FoxP3TALE-SD mCherry	hEF1α driven TALE targeting the following sequence of the human FoxP3 promoter: TATGAGAACCC CCCCCCACCCCGTGAT (chrX:49,119,959-49,122,658) fused to a KRAB silencing domain and a T2A linked mCherry marker (*piggyBac* Transposon)	Figure 4.10 B
PB tetOTALEpart-SD 1 mCherry	hEF1α driven TALE partly targeting the *tet*O fused to a KRAB silencing domain and a T2A linked mCherry marker (adapted from the previously described *tet*O targeting TALE in [92]) (*piggyBac* Transposon)	Figure 4.11
PB tetOTALEpart-SD 2 mCherry	hEF1α driven TALE partly targeting the *tet*O fused to a KRAB silencing domain and a T2A linked mCherry marker (adapted from the previously described *tet*O targeting TALE in[92]) (*piggyBac* Transposon)	Figure 4.11
iDim HA tetTALE-SD mCherry	*tet*O targeting TALE fused to a KRAB silencing domain and a T2A linked mCherry marker with a N-terminal HA tag; driven by A/C Heterodimerizer inducible promoter	Figure 4.22
SB HA tetTALE-RXE-AD mCherry	*tet*O targeting TALE fused to the RXE unit, a VP64 activation domain and a T2A linked mCherry marker with a N-terminal HA tag (*Sleeping Beauty* Transposon)	Figure 4.24/4.25
PB EF HA tetTALE-RXE-SD mCherry	hEF1α driven *tet*O targeting TALE fused to the RXE unit, a KRAB silencing domain and a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.30/4.31/4.32/ 4.33

Table 11 (continued)

name	description	used in Figure
PB HA tetTALE-RXE mCherry	CMV driven *tet*O targeting TALE without transcriptional regulatory domain fused to the RXE unit and a T2A linked mCherry marker with a N-terminal HA tag (*piggyBac* Transposon)	Figure 4.26
SB tetTALE mCherry EGFP	*tet*O targeting TALE fused to a mCherry marker instead of a transcriptional regulatory domain and a T2A linked EGFP marker with a N-terminal HA tag (*Sleeping Beauty* Transposon)	Figure 4.37 B/4.39 B
CRISPR/Cas constructs		
dCas9-AD sg tetOa mCherry	dCas9 fused to a VP64 activation domain and a T2A linked mCherry marker; sgRNA targeting the *tet*O, target a	Figure 4.50/4.53 B
dCas9-AD sg tetOb mCherry	dCas9 fused to a VP64 activation domain and a T2A linked mCherry marker; sgRNA targeting the *tet*O, target b	Figure 4.50/4.53 B
dCas9-AD sg empty mCherry	dCas9 fused to a VP64 activation domain and a T2A linked mCherry marker; no sgRNA	Figure 4.50/4.53 B
dCas9-SD sg tetOa mCherry	dCas9 fused to a KRAB silencing domain and a T2A linked mCherry marker; sgRNA targeting the *tet*O, target a	Figure 4.51
dCas9-SD sg tetOb mCherry	dCas9 fused to a KRAB silencing domain and a T2A linked mCherry marker; sgRNA targeting the *tet*O, target b	Figure 4.51
dCas9-SD sg empty mCherry	dCas9 fused to a KRAB silencing domain and a T2A linked mCherry marker; no sgRNA	Figure 4.51
dCas9 sg tetOa mCherry	dCas9 without transcriptional regulatory domain and a T2A linked mCherry marker; sgRNA targeting the *tet*O, target a	Figure 4.52

Table 11 (continued)

name	description	used in Figure
dCas9 sg tetOa mCherry	dCas9 without transcriptional regulatory domain and a T2A linked mCherry marker; sgRNA targeting the *tet*O, target b	Figure 4.52
dCas9 sg empty mCherry	dCas9 without transcriptional regulatory domain and a T2A linked mCherry marker; no sgRNA	Figure 4.52

3 Methods

3.1 Bacterial culture

3.1.1 Growth of bacterial cultures

Escherichia coli (*E. coli*) liquid cultures were carried out in LB medium at 37 °C in an orbital shaker at 200 rpm. To this end, 2-100 ml LB medium were inoculated with either a single colony or 200 µl of a pre-culture and incubated overnight.

E. coli culture on plates was done by spreading *E. coli* suspension on LB agar plates with the appropriate selection antibiotics. Incubation was over night at 37 °C.

3.1.2 Cryopreservation

For long-term storage, 250 µl of an *E. coli* suspension was grown to log phase and was mixed with 750 µl 50% glycerol and frozen at -80 °C.

3.2 Cloning procedures

3.2.1 Restriction digest

To linearize plasmid DNA or to isolate DNA fragments, restriction enzymes were used. The most commonly used type II restriction enzymes recognize palindromic DNA sequences of 4-8 base pairs and cut directly at their recognition sites. For complete digestion, 1 µg of plasmid DNA was incubated with 2-5 units of the respective enzymes for 1 hour at 37 °C (unless stated otherwise by the manufacturer) using the provided reaction buffer.

3.2.2 PCR

If no appropriate restriction sites for isolation of a region of interest were available, the sequence was amplified by PCR. Primers were designed in a way that they contained restriction sites at their 5’ end. PCR was performed using the Pwo polymerase, because its proof reading capacity minimizes the risk of introducing mutations. The PCR product was then digested with the according restriction enzymes.

Table 12: PCR protocol

Step	Time [min]	Temperature [°C]	Cycles
Denaturation	10	95	1x
Denaturation	1	95	
Annealing	1	55-65	20x
Extension	1min/ 1kb	72	
Final extension	5	72	1x

3.2.3 Gel electrophoresis

Gel electrophoresis was used to purify linearized DNA and verify the correct fragment size. Agarose gels were prepared by adding a defined amount of agarose to TAE buffer and boiling it until the agarose was completely dissolved. Ethidium bromide, which intercalates with double stranded DNA and is fluorescent when exposed to UV light, was added with 5 µl/100 ml gel. After cooling, the gel was transferred to the running chamber. The restriction digest was mixed with 6x loading buffer containing bromophenol blue and loaded to a 0.8-2% agarose gel. By applying 90 volts for 1 hour, the negatively charged DNA moves through the gel towards the anode and is separated according to size, with small fragments migrating faster than bigger ones. Fragments of the desired size were cut out with a scalpel and transferred to an Eppendorf tube.

3.2.4 DNA purification from agarose gels

To purify DNA from agarose gels, the “NucleoSpin Gel and PCR Clean-up kit” from Macherey-Nagel was used according to manufacturer’s instructions. After the addition of binding buffer, the agarose gel was dissolved at 50 °C for 5-10 minutes. The solution was then loaded to a silica membrane column and DNA was eluted after several washing steps under low salt conditions.

3.2.5 Dephosphorylation

Terminal 5’ phosphate groups from DNA backbones were removed to prevent religation when only one restriction enzyme was used for linearization or two

enzymes producing complementary cohesive ends. After the purification from the agarose gel the DNA was treated with Shrimp alkaline phosphatase (SAP).

Reaction mixture
17 µl purified DNA
2 µl 10x SuperSAP reaction buffer
1 µl Super SAP
The reaction was incubated for 5 minutes at 37 °C, followed by enzyme inactivation for 15 minutes at 65 °C.

3.2.6 Oligonucleotide annealing

All oligonucleotides were purchased from Invitrogen as a desalted lyophilisates. They were dissolved in nuclease-free water at a concentration of 100 µM. Before annealing of two complementary oligonucleotides, each was phosphorylated separately with T4 polynucleotide kinase.

Reaction mixture
3 µl Oligo (100 µM)
5 µl 10x PNK buffer
0.5 µl ATP (200 mM)
41.5 µl H_2O
1 µl T4 PNK

The reaction was incubated at 37 °C for 30 minutes, followed by enzyme inactivation at 65 °C for 15 minutes. Afterwards, the two oligonucleotides were pooled and 1 µl NaCl (5M) was added. Incubation at 90 °C for 5 minutes was followed by a slow cooling to room temperature to enable strand hybridization.

3.2.7 Ligation

DNA fragments gained through restriction digest and subsequent electrophoretic purification were used for ligation. Typically molar insert: backbone ratios of approximately 1:3 were used in all reactions.

Reaction mixture

4.5 µl insert/backbone mixture
5 µl 2x Quick DNA Ligase buffer
0.5 µl T4 Quick DNA Ligase

The reaction was incubated for 5 minutes at room temperature and then kept on ice until transformation.

3.2.8 Transformation

Chemically competent *E. coli* were incubated with the ligation mix or plasmid on ice for 30 minutes. Heat shock was done for 1 minute at 42 °C, followed by 2 minutes on ice. Afterwards LB medium without antibiotic was added and the cells were incubated for 1 hour at 37 °C and 500 rpm. After centrifugation for 1 minute at 3000 x g and removal of the supernatant, cells were spread on selective agar plates.

3.2.9 Blue-White screening

The Blue-White screening is a method used in molecular biology to facilitate the assessment of cloning results. The *lacZ* gene coding for β-galactosidase is part of the plasmid backbone. The addition of IPTG induces its expression and provided X-Gal is cleaved to 5,5'-dibromo-4,4'-dichloro-indigo, making the bacterial colony a blue color. When the *lacZ* gene is disrupted by the integration of a transgene, the colonies appear white, as no β-galactosidase activity is present [93].

3.2.10 Isolation of plasmids from *E. coli* cultures

The isolation of plasmid DNA from *E. coli* cultures was performed using the purification kits from Macherey-Nagel according to manufacturer's instruction. The kit relies on alkaline cell lysis, followed by neutralization to provide optimal conditions for plasmid binding to a silica membrane. After several washing steps the purified DNA was eluted under low ionic strength conditions.

3.2.11 Photometric DNA quantification

DNA concentrations were measured using the NanoDrop UV-Vis spectrophotometer. The absorption maximum of DNA is at 260 nm and the concentration can be determined using the Lambert-Beer law.

$$\text{concentration} = \frac{\text{Absorption}_{260\,\text{nm}}}{\text{light path} \cdot \text{extinction coefficient}}$$

Sample purity was assessed by checking the 260/280 nm ratio, which should be between 1.8 and 2 for DNA.

3.2.12 DNA sequencing

All sequencing reactions were performed by the company Seqlab in Göttingen using the Sanger sequencing technique. Sample preparation was done according to the company's requirements.

3.3 Gene synthesis

Gene synthesis was required for the integration of the Retinoid X/ecdysone gene switch in the tetTALE construct. To this end, the sequence of the retinoid X receptor-α linked to the ecdysone receptor, previously described and kindly provided by the group of Carlos F. Barbas III [94], was synthesized by GeneArt and delivered as an insert in a pMK-RQ backbone.

3.4 Construction of TAL effectors

All TAL effectors were assembled using the Golden Gate TALEN and TAL Effector kit obtained from Addgene [54]. The Golden Gate cloning strategy is based on the use of type IIS restriction enzymes, which cut outside their recognition site, enabling custom defined overhangs. For the recognition of the nucleotides A, T, G and C, the repeats NI, NG, NK and HD were used, respectively. The modules were first assembled in blocks of up to ten. In the final step, those blocks and the last half repeat were brought together in the expression vector. The expression vector contained the N- and C-terminal TALE region from pthXo1, including the original nuclear localization site (NLS) but not the plant activation domain as described by

Cermak *et al*. [54,95]. The C-terminal part of the backbones was furthermore modified such that the NLS from simian virus 40 large T antigen [96] and a regulatory domain was included. This domain was either the C-terminal end of the human Kox1 zinc finger protein containing the KRAB domain (SD) [20] or the herpes simplex viral protein VP16 (AD) [97]. For expression monitoring, a N-terminal HA tag was introduced and either a EGFP or a mCherry fluorescent protein was linked via a T2A site.

3.5 Methylation analysis

3.5.1 Isolation of genomic DNA

Isolation of genomic DNA from eukaryotic cells was performed with the “NucleoSpin Tissue kit” from Macherey-Nagel according to manufacturer’s instruction. Cells are lysed by incubation with Proteinase K and SDS. By addition of ethanol and chaotropic salts the genomic DNA was prepared for binding to the silica membrane column. After several washing steps pure DNA is eluted under low ionic strength conditions.

3.5.2 Bisulfite conversion

The EpiTect Bisulfite Kit was used for bisulfite conversion of genomic DNA for methylation analysis according to manufacturer’s instructions. 1 µg of genomic DNA was used per reaction. The DNA is incubated with bisulfite salt at high temperatures and low pH, which results in the deamination of unmethylated cytosines to uracil, subsequently PCR-amplified as thymines. The methyl group of methylated cytosines protects it from conversion and enables discrimination.

3.5.3 PCR amplification of bisulfite converted DNA

50 ng of bisulfite converted genomic DNA was used for PCR amplification of the promoter region of interest. The HotStarTaq polymerase from QIAGEN was used according to manufacturer’s instructions. The appropriate annealing temperature was empirically tested for each amplicon using a temperature gradient. PCR products were purified through gel electrophoresis. The Taq polymerase adds an adenosine at the 3’ end of the PCR product [98], which can be used for subsequent TA cloning.

3.5.4 TA cloning of PCR amplicons

The pMD20 vector supplied by TAKARA was used for the cloning of PCR products with 3'-terminal adenosine overhangs. This linearized vector has matching 3'-terminal thymidine overhangs and the successful cloning was checked through blue white screening.

Reaction mixture

0.5 µl linearized pMD20 (50 ng/µl)
4 µl purified PCR product
5 µl 2x Quick DNA Ligase buffer
0.5 µl T4 Quick DNA Ligase

3.6 Cell culture

All cell culture procedures were performed in a laminar flow cabinet using sterile equipment.

3.6.1 Maintenance of cells

HAFTL cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS and 50 mM β-mercaptoethanol. Selection of stably transfected pools and clones was achieved by addition of 20 µg/ml blasticidin.
Maintenance of HeLa (**ATCC:**CCL-2) and the HeLa-derived stable cell lines X1/5 and X1/6 was done in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS. Selection was achieved by addition of 5 µg/ml of blasticidin or 2 µg/ml of puromycin. Cells were cultured with 50-100 ng/ml dox where indicated.
CHO K1 and CHO Tet-On Advanced cells were cultivated in RPMI 1640 medium supplemented with 10% FCS. Antibiotic selection was performed with 5 µg/ml of blasticidin or 10 µg/ml of puromycin. Cells were cultured with 1 µg/ml dox where indicated.
HEK 293TN and HEK 293 Tet-On Advanced cells were maintained in DMEM with 10% FCS, 10 mM non-essential amino acids and 1 mM sodium pyruvate. Selection was achieved by addition of 10 µg/ml of blasticidin or 4 µg/ml of puromycin. Cells were cultured with 1 µg/ml dox where indicated.

All cells were maintained in medium supplemented with 200 mM of L-glutamine (except HEK 293 cells, where 400 mM were used) and 100 units/ml of penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified incubator.

3.6.2 Cell passaging

Adherent cells were passaged when reaching a confluency of approximately 80%. After washing with PBS, TrypLE was added for dissociation and the cells were incubated for 5 minutes at room temperature. After checking the dissociation by gentle shaking, fresh medium was added and the cells were thoroughly resuspended. A portion of the cells was transferred to a new cell culture flask with fresh medium. In case of a cell density lower than 80%, medium was changed at least every 2-3 days.
Suspension cells were carefully mixed and a fraction was transferred to a new cell culture flask with fresh medium.

3.6.3 Cell counting

Cells were harvested for normal passaging and 10 µl of the suspension was transferred to a 96 well plate. Another 10 µl of 0.4% trypan blue solution was added to assess cell viability. Intact cells appear colorless, whereas the dye enters dead or perforated cells, which then appear dark blue. The mixture was transferred to an Improved Neubauer haemocytometer. Living cells were counted in four quadrants and the cell number was calculated according to the following equation:

$$\frac{\text{cell count}}{4} \cdot 10^4 \cdot \text{dilution factor} = \text{cells/ml}$$

3.6.4 Freezing and thawing of cells

Cells were grown to confluency before freezing and treated like described for cell passage. After cell counting and centrifugation for 5 minutes at 300 x g, cells were resuspended at a concentration of $1 \cdot 10^6$ cells/400 µl and transferred into a cryotube with 100 µl DMSO and 500 µl FCS. For controlled freezing with a decrease in temperature of 1 °C/min, cells were stored in a Mr. Frosty freezing container at -80 °C for at least 12 hours. The tubes were then transferred to liquid nitrogen for long-term storage.

For thawing the cells, the tubes were transferred from liquid nitrogen into a 37 °C water bath. The total volume of 1 ml was added to 9 ml of fresh culture medium and thoroughly mixed. After centrifugation for 5 minutes at 300 x g, cells were resuspended in 5 ml medium and transferred to a T25 cell culture flask. If addition of antibiotics was necessary, this was done 24 hours after thawing.

3.6.5 Isolation of single clones

Clonal selection was achieved by following a limited dilution approach for all cell lines. Cells were harvested and the cell number was determined. By serial dilution the concentration was adjusted to 5 cells/1 ml for adherent cells and 2.5 cells/1 ml for suspension cells. 200 µl of that solution was then added to each well of a flat bottom 96 well plate. After 24 hours, the plate was screened for single cells in the wells. Single clones were then grown to confluency and transferred to a larger format.

3.6.6 Density gradient centrifugation

In some cases cell viability drastically decreased after electroporation or during antibiotic selection. To purify the viable cells from cell debris, a density gradient was performed. To this end, 3 ml of the Ficoll-based LSM 1077 solution, with a density of 1077 g/ml of a saccharose polymer, was pipetted in a 15 ml Falcon tube. The cell suspension was cautiously pipetted on top, taking care to avoid mixing. Centrifugation for 20 minutes at 800 x g without activating the centrifuge brake resulted in pelleting of cell debris at the bottom of the tube. Viable cells were located at the interface between the LSM 1077 solution and the medium and were carefully transferred into a fresh 15 ml Falcon tube. After washing with 10 ml of fresh medium the cells were seeded again in a cell culture dish of appropriate size.

3.6.7 Transfection and transposition

HAFTL cells were transfected by electroporation. $1 \cdot 10^7$ cells were resuspended in 400 µl of antibiotic free medium and mixed with 10 µg of plasmid DNA plus 30 µg of salmon sperm DNA. After incubation for 20 minutes at room temperature, the cells were transferred to a 0.4 cm cuvette and electroporated with the Gene Pulser Xcell from Biorad at 300 volts and 950 µF using a single exponentially decaying pulse. After another 15 minutes incubation at room temperature, the cells were seeded with

fresh medium without antibiotics. The same procedure was applied for Jurkat cells, using $1 \cdot 10^6$ cells and electroporation conditions of 180 volts and 950 µF.
Adherent cells were transfected using polyethylenimine PEI [99]. 4 µl of 7.5 mM PEI solution was mixed with 50 µl of 150 mM NaCl and then added to a total of 1 µg of plasmid DNA in 50 µl of 150 mM NaCl. After vortexing, the mixture was incubated for 10 minutes at room temperature and then added to 50% confluent cells in a 6 well plate.
For stable cell line generation, expression units were chromosomally integrated via transposition. pT2-based *Sleeping Beauty* transposons [100], containing the human elongation factor 1 alpha promoter (hEF1α) and a puromycin selection cassette, were used in conjunction with SB100 hyperactive transposase [101]. Alternatively, pPB-based *piggyBac* transposons [102], containing hEF1α promoter and a blasticidin selection cassette, were used in conjunction with optimized mPB transposase [103].

3.6.8 Viral packaging

Viruses were packaged using HEK293 TN cells and the packaging plasmids from the Trono lab, CH, distributed by Addgene. Cells were grown in a 6 well plate to 50% confluency and then transfected as follows:

0.6 µg transfer plasmid (encoding the cDNA sequence to be stably expressed)
0.6 µg psPAX2 (gag and pol)
0.6 µg pMD2.G (coding for the VSV-G glycoprotein)

The medium was changed six hours after transfection. The supernatant was harvested on day 2 and 3 after transfection and concentrated using the Lenti-X Concentrator. 4 ml of the concentrator was added to a total volume of 12 ml supernatant. After incubation for 1 hour at 4 °C, the mixture was centrifuged for 45 minutes at 1500 x g. The supernatant was discarded and the pellet containing the viral particles was resuspended in 1 ml PBS. Small aliquots were stored at -80 °C.

3.6.9 Virus titer determination

The viral titer was determined by transducing HEK 293 TN cells. $5 \cdot 10^4$ cells per well were seeded in a 12 well plate one day prior to transduction. 24 hours after seeding,

the medium was changed and the cell number was determined to enable exact calculations. The medium was supplemented with 8 µg/ml polybrene, which neutralizes the negative charges of the cell and virus surface and thereby facilitates infection [104]. For transduction, 200 µl, 20 µl, 80 µl and 1.6 µl of the concentrated virus supernatant were added to the cells and EGFP reporter expression was determined by FACS 72 hours later. The number of viral particles was calculated with the following formula:

$$\frac{\text{transduction unit}}{\text{ml}} = \frac{\text{number of transduced cells} \cdot \frac{\%\ \text{EGFP}^{+}\text{cells}}{100}}{\text{Volume of virus supernatent in ml}}$$

3.6.10 Transduction for single copy integration

Transduction of cells in a way that only a single copy of the transfer plasmid is integrated, requires a low multiplicity of infection (MOI). To this end, the cell number was determined and the amount of viral supernatant was chosen in a way that on average 0.1 viral particles per cell were applied. The medium was changed 24 hours after transduction, and selection was started another 24 hours later.

3.6.11 Cell sorting

For cell sorting, cells were grown to 80% confluency and harvested as described for passaging. After centrifugation for 5 minutes at 300 x g the cell pellet was taken up in FACS buffer and filtered through a 35 µm nylon mesh to ensure a single cell suspension. The cells were then transferred to the BCRT flow cytometry lab where the BD FACSAria™ III was used to sort the cells according to their fluorescence intensity.

3.7 Analytical methods

3.7.1 Luciferase assay

Luciferase reporter assays are a very sensitive tool to determine promoter activities. They are performed by providing the enzyme-specific substrate and measuring the light emitted during the catalytic reaction. To this end, cells transfected with a firefly luciferase reporter were lysed and 5% of the lysate was added to 90 µl reaction buffer. The injection of 50 µl substrate buffer was done automatically 0.5 seconds

before the measurement in the Mithras LB 940 microplate reader. A constitutively active *Renilla* luciferase construct was co-transfected in transient reporter assays for internal standardization. Measurements were performed analogously with the exception of the substrate buffer that contained coelenterazine instead of luciferin.

3.7.2 BCA assay

For experiments with cells carrying chromosomal copies of different luciferase reporters, firefly luciferase activity was normalized to the protein content. The protein concentration was measured using a BCA assay kit. It is based on the reduction of Cu^{2+} to Cu^{+} by peptides and the subsequent chelating of bicinchoninic acid with Cu^{+} forming a purple product [105]. The assay was performed according to manufacturer's instructions.

3.7.3 Flow cytometry

The expression of fluorescent proteins was measured using a flow cytometer. With this method single cells are passed through laser beams of different wavelengths and resulting emission from fluorescent proteins is detected. Moreover, data about cell size and granularity is collected. Before analysis, cells were harvested as described for cell passaging. After a centrifugation step at 300 x g for 5 minutes, cells were resuspended in FACS buffer and filtered through a 35 µm nylon mesh to ensure a single cell suspension. For the detection of EGFP, the BD Accuri cytometer was used. The BD LSRII flow cytometer was used for simultaneous detection of EGFP and mCherry.

3.7.4 Immunoblotting

Immunoblotting was used to detect HA-tagged tetTALE protein. With this method proteins are first separated according to their molecular weight in an acrylamide gel and then transferred to a PVDF or nitrocellulose membrane. The incubation with an epitope-specific primary antibody and subsequent treatment with a labeled secondary antibody allows the detection of defined proteins.
Cells were harvested either by trypsinization (adherent cells) or centrifugation (suspension cells) and washed twice with PBS. Afterwards, the cells were directly lysed in 2x loading buffer and the genomic DNA was sheared with a syringe. All

samples were incubated for 10 minutes at 70°C before loading on a NuPAGE Novex 4-12% Bis-Tris gradient gel. Mighty Small wet blotting system was used to transfer proteins to a nitrocellulose membrane. The transfer was performed for 1 hour at 400 mA with constant cooling. After transfer, the blot was blocked for one hour with Odyssey blocking buffer. For detection of HA-tagged proteins, a rabbit anti-HA antibody was used. As a reference, β-actin was detected with a mouse anti β-actin antibody. Incubation of the blot with the first antibody was done at 4°C overnight in blocking buffer. After three washing steps with TBS-T, the blot was incubated for 1 h at room temperature with an anti-rabbit antibody coupled to the IRDye 800CW as well as an anti-mouse antibody coupled to IRDye 680, followed by two washes in TBS-T and one in TBS. Blot analysis was done on the Odyssey infrared imaging system (Licor) using the manufacturer's software.

3.7.5 Microscopy

Fluorescence microscopy, to assess transfection efficiency or cell density, was performed using the Leica DM-IL microscope. For documentation, high quality pictures were taken using the Axio Observer from Zeiss.

4 Results

4.1 Activation with TAL Effectors

4.1.1 TAL effectors as efficient transcriptional activators

TAL effectors have emerged as a valuable and widely accepted tool for targeting DNA and bringing functional domains like transcriptional activators or repressors close to their desired site of action. Compared to the well-established zinc fingers [106,107], relatively few studies about TALEs have focused on the careful characterization of their mode of action and possible optimizations.

One apparent way of doing so is to compare TALEs to pre-existing systems. In this thesis the Tet system was chosen for comparison considering the large amount of pre-characterized constructs and cell lines. TAL effectors targeting the 19 bp tetO2 sequence were constructed (tetTALE). Their binding site is identical to that of all tetR-based transcription factors, allowing a direct comparison to the well-established Tet system. Extensive functional testing and characterization was done in activation assays. These are better suited to determine little variability in performance when compared to repression assays, as basal levels of reporter expression are low and even moderate changes in expression are readily detectable. For its high sensitivity a luciferase reporter was used, driven by a tet-responsive promoter with one or seven copies of the tet operator (P_{tet1} luc or P_{tet7} luc [30]). TetTALE, fused to a VP64 activation domain (tetTALE-AD), was used in all activation experiments (see Figure 4.1 A). For comparison, a tTAs construct was employed coding for the tetracycline-induced tet *trans*-activator [30].

First, the optimal ratio of reporter to tetTALE-AD was tested in a double transient transfection experiment in HeLa cells. As shown in Figure 4.1 B, activation by tetTALE-AD was observed over a broad range of ratios with a maximum of 650-fold and was comparable to tTAs-induced activation. All further experiments were performed with a ratio of 1:1 (w/w) unless stated otherwise.

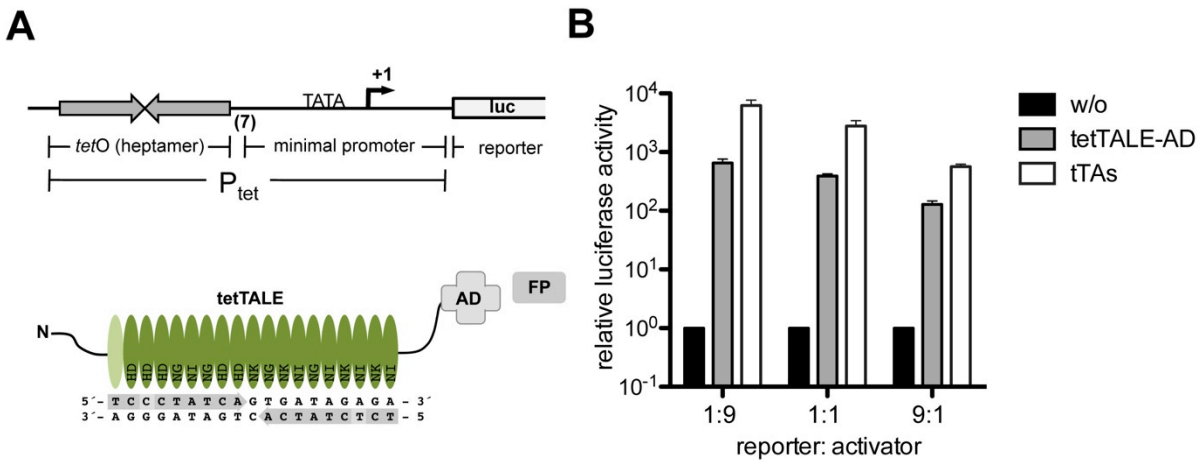

Figure 4.1 Activation capacity of tetTALE-AD over a broad range of reporter to activator ratios
(A) The activation reporter consists of a firefly luciferase gene driven by a tet-responsive promoter carrying either one or seven copies of the tet operator (*tet*O) (upper panel). TetTALE was constructed to target the 19 bp *tet*O sequence. The DNA-binding domain with the target sequence and the used RVDs is depicted. Light grey boxes mark positions deviating from the operator symmetry. The C-terminus encompasses a nuclear localization signal and a VP64 acivation domain (AD). The fluorescence protein (FP) is linked via a T2A site (lower panel).
(B) HeLa cells were co-transfected with P_{tet7} luc and either a tTAs or a tetTALE-AD expression construct in different ratios. A *Renilla* luciferase expression construct was included for internal standardization. P_{tet7} activity without activator was set to 1. Shown are mean values of three independent transfections with standard deviation.
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To get a better impression of the activation capacity on the single cell level, activation assays were also performed with a d2EGFP reporter driven by a tet-responsive promoter. Again comparable activation by tetTALE-AD and tTAs was observed with similar intensity distribution of the d2GFP$^+$ population, showing that also the maximum activation level was alike (see Figure 4.2 A).

As has been reported several times, the recruitment of multiple activation domains to a promoter results in synergistic transcriptional activation [12]. To assess this for our tetTALE-AD, reporters carrying one (P_{tet1} luc) or seven (P_{tet7} luc) copies of the *tet*O target were used. As detected for the tTAs, tetTALE-AD-mediated activation increased considerably (40 fold) with seven compared to a single target site, clearly arguing for a synergistic mechanism. As expected, tetTALE-AD activity was not dependent on the Tet system inducer dox (see Figure 4.2 B).

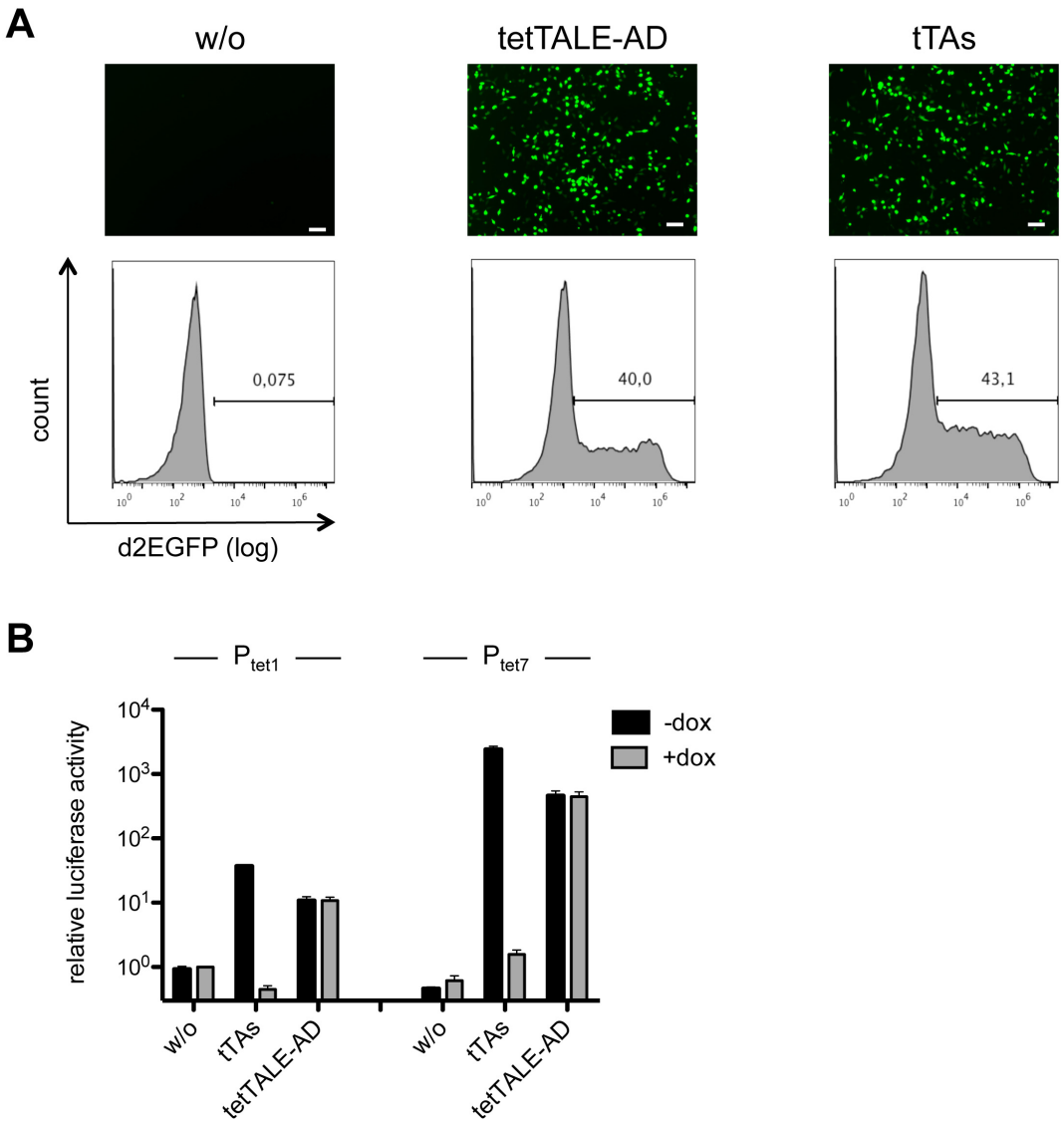

Figure 4.2 Synergistic effect for tetTALE-AD-mediated activation
(A) HeLa cells were co-transfected with a P_{tet7} d2EGFP reporter construct and either a tTAs or a tetTALE-AD expression construct. Microscopic pictures were taken 24h after transfection. Scale bar: 100 µm. FACS analysis was performed 48h after transfection. Cells transfected with the reporter only (w/o) served as a control.
(B) HeLa cells were co-transfected with P_{tet1} luc or P_{tet7} luc and either a tTAs or a tetTALE-AD expression construct. A *Renilla* luciferase expression construct was included for internal standardization. P_{tet1} activity in the presence of doxycycline (dox) was set to 1. Shown are mean values of three independent transfections with standard deviation.
Partly published in Werner & Gossen, 2014 [108] target was evaluated. X1/6 [33] cells carrying chromosomal copies of P_{tet7} luc were

The particular benefit of TALEs as transcriptional regulators is their applicability for targeting endogenous loci. Therefore, the activity of tetTALE-AD on a chromosomal target was evaluated. X1/6 cells [33], carrying chromosomal copies of P_{tet7}, were transfected with either tetTALE-AD or tTAs. As shown in Figure 4.3, tetTALE-AD induced activity of the chromosomal reporter by more than 2000-fold, a level also

observed for tTAs, demonstrating its applicability beyond transient assays. This is a prerequisite for the analysis of modes of repression, which will be focused on later.

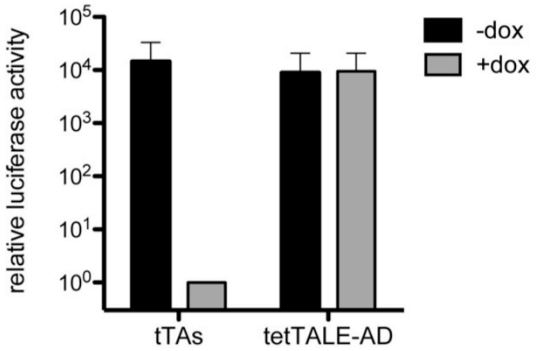

Figure 4.3 Activation of a chromosomal target by tetTALE-AD
X1/6 cells, carrying the P_{tet7} luc reporter stably integrated in the chromosome, were transfected with either a tTAs or a tetTALE-AD expression construct. A *Renilla* luciferase expression construct was included for internal standardization. P_{tet7} activity in the presence of doxycycline (dox) was set to 1. Shown are mean values of three independent transfections with standard deviation.
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4.1.2 Orientation dependence of TALE-mediated activation

Several different architectures for TALEs are available, differing among other things in the position of the activation domain relative to the transcriptional start site (TSS). The constructs P_{tet7} luc and $P_{tet7(rev)}$ luc, carrying the *tet*O or *tet*O(rev) ([30] published there as pUHC 13-3 and pUHC 13-4, respectively), were used to investigate this aspect. As shown in Figure 4.4, the orientation of the tetTALE-AD had no influence on transcriptional activation. Naturally the same was true for tTAs because as a dimer the orientation does not change by reversing the target sequence.

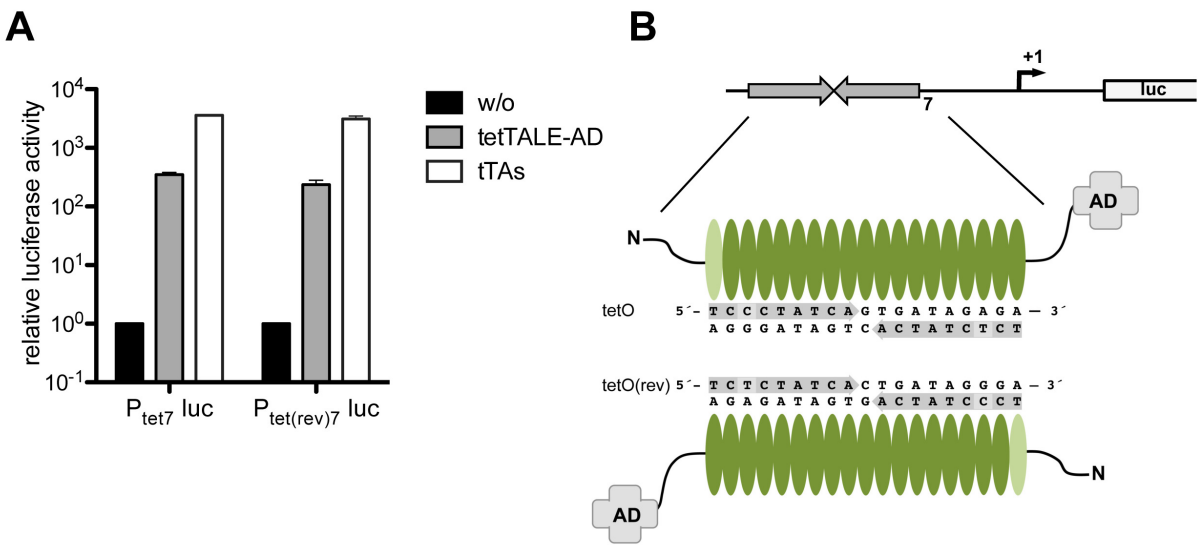

Figure 4.4 Dependence of tetTALE-AD-mediated activation on orientation relative to the TSS
(A) HeLa cells were co-transfected with P_{tet7} luc or $P_{tet(rev)7}$ luc and either a tTAs or a tetTALE-AD expression construct. A *Renilla* luciferase expression construct was included for internal standardization. P_{tet7} and $P_{tet(rev)7}$ activity without activator was set to 1. Shown are mean values of three independent transfections with standard deviation
(B) Schematic representation of the *tet*O or *tet*O(rev) reporter construct with bound tetTALE-AD. Light grey boxes mark positions deviating from the operator symmetry.

4.1.3 Influence of mismatches in the target sequence on tetTALE DNA-binding

To test the sensitivity of tetTALE-AD activity to mismatches in its target sequence, pre-existing constructs with two mutations in the *tet*O sequence were used [109]. Interestingly, depending on the position and the context of the mismatches, the activity was either largely unchanged (*tet*O 4C) or dramatically decreased (*tet*O 6C) (see Figure 4.5). When the mismatches were located more to the 5’ or 3’ end of the target sequence, activation capacity was completely abrogated, whereas more central mismatches only led to a decrease by a factor of 2.5. Activation by tTAs was assessed in the same experiment but as it binds as a dimer, each molecule only encounters a single mismatch, limiting the comparability. Activation by tTAs did not show these pronounced effects but a rather moderate decrease in activation, however, with the same tendency.

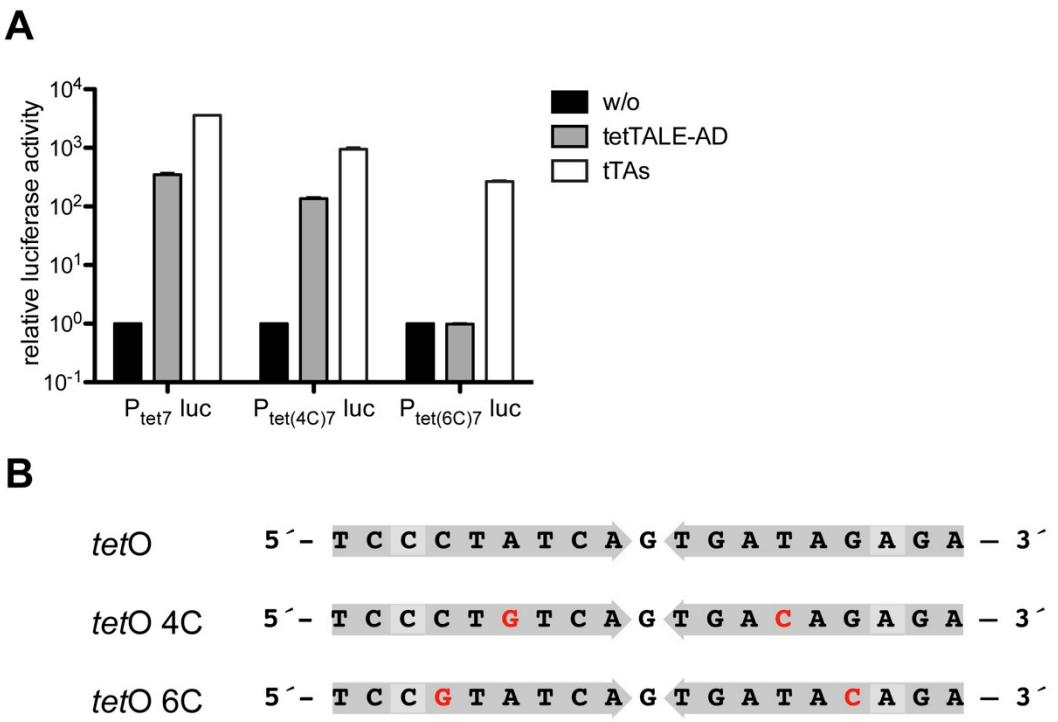

Figure 4.5 Activation capacity of tetTALE-AD compared to tTAs using different *tet*O variants
(A) HeLa cells were co-transfected with luciferase reporter constructs carrying variants of P_{tet7} with two mutations in the *tet*O sequence and either a tTAs or a tetTALE-AD expression construct. A *Renilla* luciferase expression construct was included for internal standardization. Luciferase activity resulting from reporters transfected without activator was set to 1. Shown are mean values of three independent transfections with standard deviation.
(B) Schematic representation of the *tet*O variants. Deviations from the original *tet*O are marked in red. Light grey boxes mark positions deviating from the operator symmetry.
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As off-target activity is of importance for the use of designer DNA-binding proteins in general and the experiment using *tet*O variants gave inconsistent results, new tetTALE-AD constructs were designed with the mismatches positioned directly in the N- or C-terminus. In one TALE the mandatory first repeat targeting the T did not match (tetTALE first Tmm). The other TALE carried five additional C-terminal repeats not matching the sequence (tetTALE 5mm) (see Figure 4.6 B). Both were tested on P_{tet7} luc in comparison to the perfect matching tetTALE-AD in a double transient experiment. None of the mismatch variants totally abolished activation, but reporter activity was moderately reduced. The C-terminal “overhang” of five TALE repeats caused a drop in activation by a factor of 3.5, whereas when the mandatory first repeat did not fit, a loss of activation by a factor of 2.7 was observed (see Figure 4.6 A).

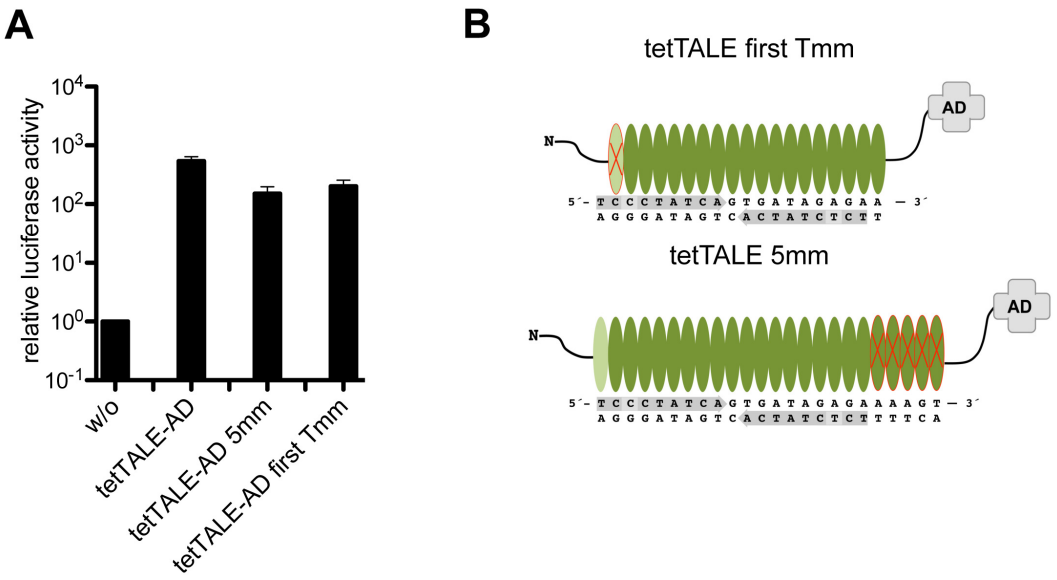

Figure 4.6 Tolerance of tetTALE-AD activation to N- and C-terminal mismatches
(A) HeLa cells were co-transfected with P_{tet7} luc and either tetTALE-AD or a tetTALE-AD construct comprising mismatching repeats. A *Renilla* luciferase expression construct was included for internal standardization. Luciferase activity resulting from P_{tet7} luc transfected without activator was set to 1. Shown are mean values of six independent transfections with standard deviation.
(B) Schematic representation of the *tet*O sequence and the tetTALE-AD constructs with mismatching repeats. Light grey boxes mark positions deviating from the operator symmetry.

One can conclude that mismatches can, but not necessarily have to, completely inhibit TALE-AD-mediated activation. The effect is apparently context-dependent.

4.1.4 Comparison of different TALE backbones used throughout the thesis

To meet the requirements of different experiments, the backbone carrying the tetTALE expression cassette was adjusted several times. It was transferred to either *Sleeping Beauty* or *piggyBac* transposon backbones to facilitate stable integration. Moreover, a HA-tag was integrated to allow detection of the tetTALE protein. To make sure these modifications had no influence on the activity, all variants were analyzed side-by-side in an activation assay. As shown in Figure 4.7, neither the terminal repeats of the transposon backbones nor the introduction of a HA-Tag had an impact on tetTALE-AD function.

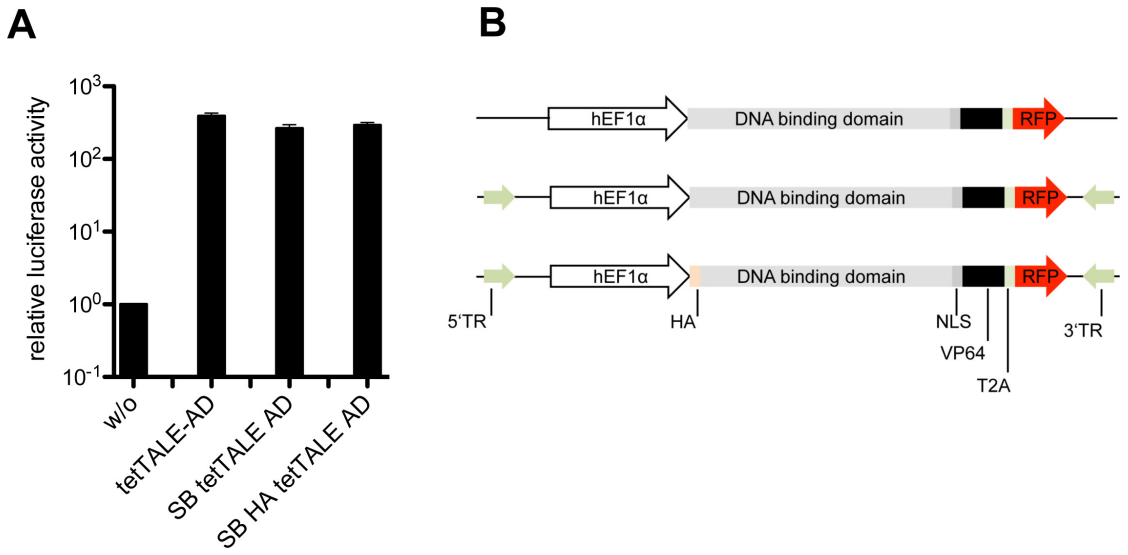

Figure 4.7. Comparison of tetTALE-AD activation in different backbones
(A) HeLa cells were co-transfected with the reporter construct P_{tet7} luc and tetTALE-AD expression cassettes in different backbones. *Renilla* luciferase expression construct was included for internal standardization. Luciferase activity resulting from P_{tet7} luc transfected without activator was set to 1. Shown are mean values of three independent transfections with standard deviation.
(B) Schematic representation of the tetTALE-AD expression construct in different backbones containing transponson terminal repeats (TR), a hemagglutinin (HA) tag or both.

4.1.5 Comparison of tetTALE activation capacity to previously described *tet*O-targeting TALEs

During the course of the thesis, TALEs partially targeting the *tet*O sequence were published [92]. To put the results into context, TALEs targeting the previously described regions of *tet*O were constructed (with little adaptations) and compared their performance to that of our construct. On the one hand the adaptions comprised the usage of NK instead of NN as published, for consistency with our tetTALE. On the other hand the last repeat of $tetOTALE_{part}2$ was changed from HD to NI, to fit our reporter constructs (see Figure 4.8 B). The transient activation assay showed highly similar results for our tetTALE-AD compared to the earlier reported tetOTALEs (see Figure 4.8 A), suggesting an overall comparability of tetTALEs described here and those by Li *et al.* [92].

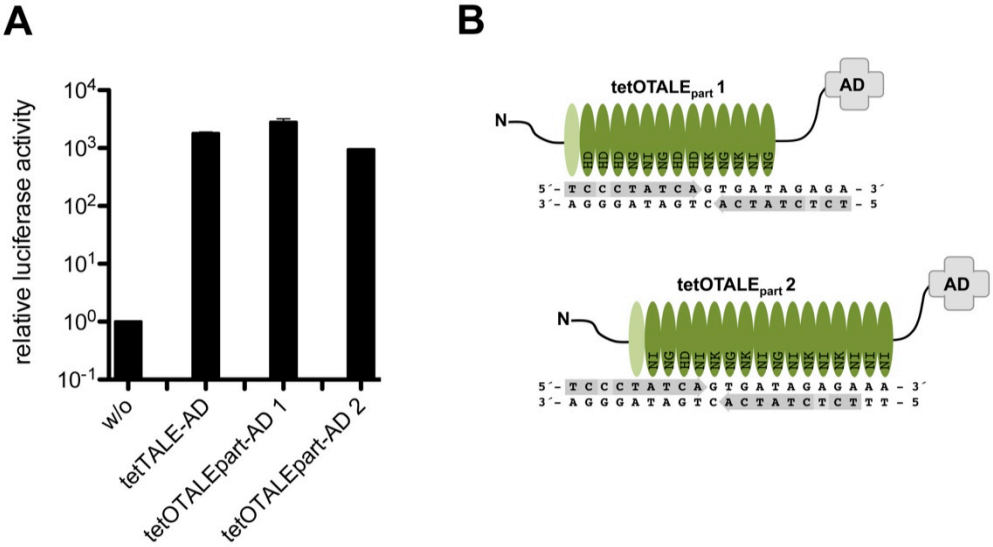

Figure 4.8 Comparison of tetTALE-AD activation to previously reported tet-promoter targeting TALEs
(A) HeLa cells were co-transfected with the reporter construct P_{tet7} luc and either tetTALE-AD or two other tet promoter-binding TALE-AD constructs partially covering the *tet*O sequence. *Renilla* luciferase expression construct was included for internal standardization. Luciferase activity resulting from P_{tet7} luc transfected without activator was set to 1. Shown are mean values of three independent transfections with standard deviation.
(B) Target sequence of the DNA-binding domains of the two tetOTALE$_{part}$ constructs according to Li *et al*. [92] and the used RVDs. Light grey boxes mark positions deviating from the operator symmetry.
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4.2 Repression with TAL Effectors *in trans*

4.2.1 TAL effectors as effective transcriptional silencers

After the thorough investigation of functionality and binding characteristics in activation assays, a tetTALE repressor was constructed to analyze the different modes of repression and their efficacy. To this end, the VP64 domain used so far was replaced by the mammalian KRAB silencing domain, previously shown to function when fused to artificial DBDs [110,111]. This resulted in tetTALE-SD as depicted in Figure 4.9 A. The pUHC13-13 construct was used as a repression reporter [112], harboring a constitutively active *tet*O modified CMV promoter driving a luciferase reporter. Repression is highly dependent on the degree of operator occupation by effectors. Therefore, different ratios of reporter to tetTALE-SD were tested in a transient transfection experiment. In accordance with the results from the analogous activation experiment, repression by tetTALE-SD was observed over a broad range of ratios, even when the reporter was in excess, with a maximum of 40-fold repression. This clearly argues for a strong repression capacity. All further experiments were performed with a ratio of 1:1 (w/w) unless stated otherwise.

Furthermore, the direct comparison to the potent tet *trans*-silencer (tetR-SD) demonstrated that tetTALE-SD's silencing capacity is similar (see Figure 4.9 B).

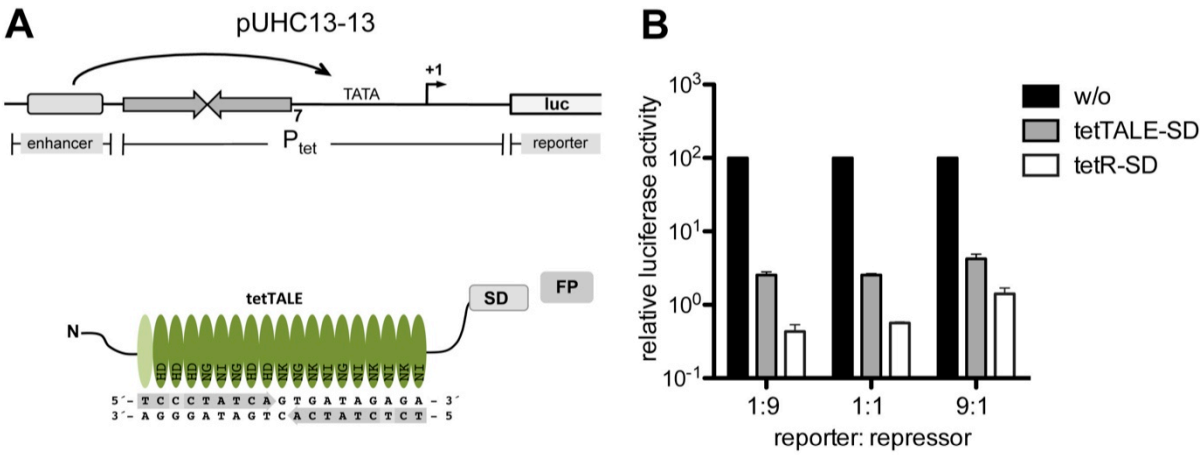

Figure 4.9 Repression capacity of tetTALE-SD over a broad range of reporter to repressor ratios
(A) The repression reporter pUHC13-13 consisted of a firefly luciferase gene driven by a constitutively active *tet*O modified CMV promoter (upper panel). TetTALE was constructed to target the 19 bp *tet*O sequence. The DNA-binding domain of tetTALE-SD with the target sequence and the used RVDs is depicted. Light grey boxes mark positions deviating from the operator symmetry. The C-terminus encompasses a nuclear localization signal and a KRAB silencing domain (SD). The fluorescence protein (FP) is linked via a T2A site (lower panel).
(B) HeLa cells were co-transfected with pUHC13-13 and either a tetR-SD or a tetTALE-SD expression construct in different ratios. A *Renilla* luciferase expression construct was included for internal standardization. Reporter activity without repressor was set to 100. Shown are mean values of three independent transfections with standard deviation.
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4.2.2 Dependence of tetTALE-SD-mediated *trans*-repression on the silencing domain and the target site

To make sure that the presence of the TALE DNA-binding domain alone does not influence reporter expression, a tetTALE without regulatory domain was constructed and tested in parallel with tetTALE-SD. As shown in Figure 4.10 A, tetTALE alone did not inhibit reporter expression in a setting where the binding site is located outside of the core promoter. Moreover, it was tested if the observed repression is a target site-specific phenomenon or a result of transfecting a transcriptional silencer per se. Therefore, a TALE-SD with an unrelated target sequence, namely the human FoxP3 promoter, was used. As expected, the reporter activity was not influenced, demonstrating a target-specific action of tetTALE-SD, which relies on the presence of the silencing domain.

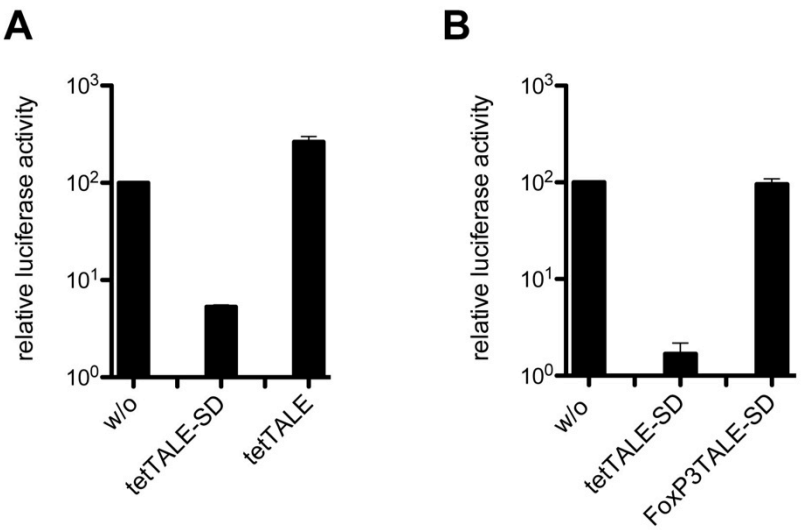

Figure 4.10 Dependence of tetTALE-SD-mediated *trans*-repression on the silencing domain and the correct target site
(A) HeLa cells were co-transfected with pUHC13-13 and either a tetTALE-SD or a tetTALE expression construct without silencing domain. A *Renilla* luciferase expression construct was included for internal standardization. Reporter activity without repressor was set to 100. Shown are mean values of three independent transfections with standard deviation.
(B) HeLa cells were co-transfected with pUHC13-13 and either a tetTALE-SD or a FoxP3TALE-SD expression construct, targeting a sequence the human FoxP3 promoter. A *Renilla* luciferase expression construct was included for internal standardization. Reporter activity without repressor was set to 100. Shown are mean values of three independent transfections with standard deviation.
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4.2.3 Comparison of tetTALE-SD repression capacity to previously described *tet*O targeting TALEs

Following the same line of argument as for the activation studies, a comparison of our tetTALE to the tetOTALE$_{part}$ constructs (see Figure 4.8 B) for *trans*-repression was performed. Like with the activation studies, tetTALE performance was similar to that of the described tet-promoter targeting TALEs (see Figure 4.11).

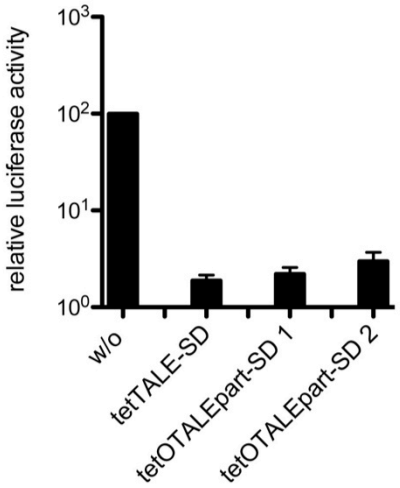

Figure 4.11 Comparison of tetTALE-SD repression to previously reported tet-promoter binding tetOTALEs
HeLa cells were co-transfected with pUHC13-13 and either tetTALE-SD or two other tet promoter-binding TALE-SD constructs partially covering the *tet*O sequence (see Figure 4.8 B). A *Renilla* luciferase expression construct was included for internal standardization. Luciferase activity resulting from the reporter alone was set to 100. Shown are mean values of three independent transfections with standard deviation. Partly published in Werner & Gossen, 2014 [108]

4.2.4 Stable repression *in trans* with tetTALE-SD

Having established that tetTALE-SD can effectively repress CMV driven expression in transient transfections, the silencer's performance on a chromosomally integrated mammalian promoter under stable expression conditions was analyzed. A previously established HAFTL cell line was used (M. Hofstätter and M. Gossen, unpublished). HAFTL cells are murine pre-B cells [90] and were used because of their favorable growth kinetics, facilitating stable expression experiments (requiring long antibiotic selection periods) as well as single clone analysis following a limited dilution approach. A destabilized ZsGreen reporter, driven by the constitutively active strong human EF1α promoter with an upstream tet-responsive element (tetEF-ZsGreen), was stably integrated in the cells by transposition. The ZsGreen reporter was destabilized by a C-terminal PEST degradation domain, making it particularly suitable for repression experiments. After clonal selection, the stable transfection resulted in a high expressing ZsGreen$^+$ cell line. Subsequently, the cells were stably transfected with either tetTALE-SD or tetTALE without silencing domain, both linked to a mCherry fluorescence reporter (see Figure 4.12). ZsGreen and tetTALE linked mCherry expression was monitored via FACS.

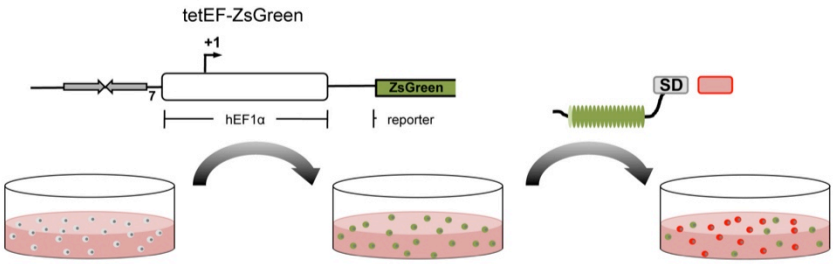

Figure 4.12 Schematic outline of the stable repression experiment
HAFTL cells were stably transfected with a hEF1α driven ZsGreen reporter construct with an upstream tet-responsive element. Single clone selection resulted in a high expressing ZsGreen^{+} cell line (middle). These cells were then stably transfected with a tetTALE-SD or tetTALE construct as a control (right).

Analysis of the cell pool showed that the clear majority of cells expressing tetTALE-SD, as monitored via mCherry signal, displayed only background ZsGreen expression. This was not observed in cells that were transfected with tetTALE alone (see Figure 4.13 upper panel). Thus the recruitment of the KRAB silencing domain to a region upstream of a strong human promoter can totally abrogate its activity.

To show that this effect is not cell line specific, three additional cell lines were created carrying tetEF-ZsGreen, namely Jurkat, HeLa and CHO, of human and rodent origin. Transfection of tetTALE-SD resulted in the repression of the ZsGreen reporter to background level in all of these cell lines, only differing in efficiency (see Figure 4.13 lower 3 panels).

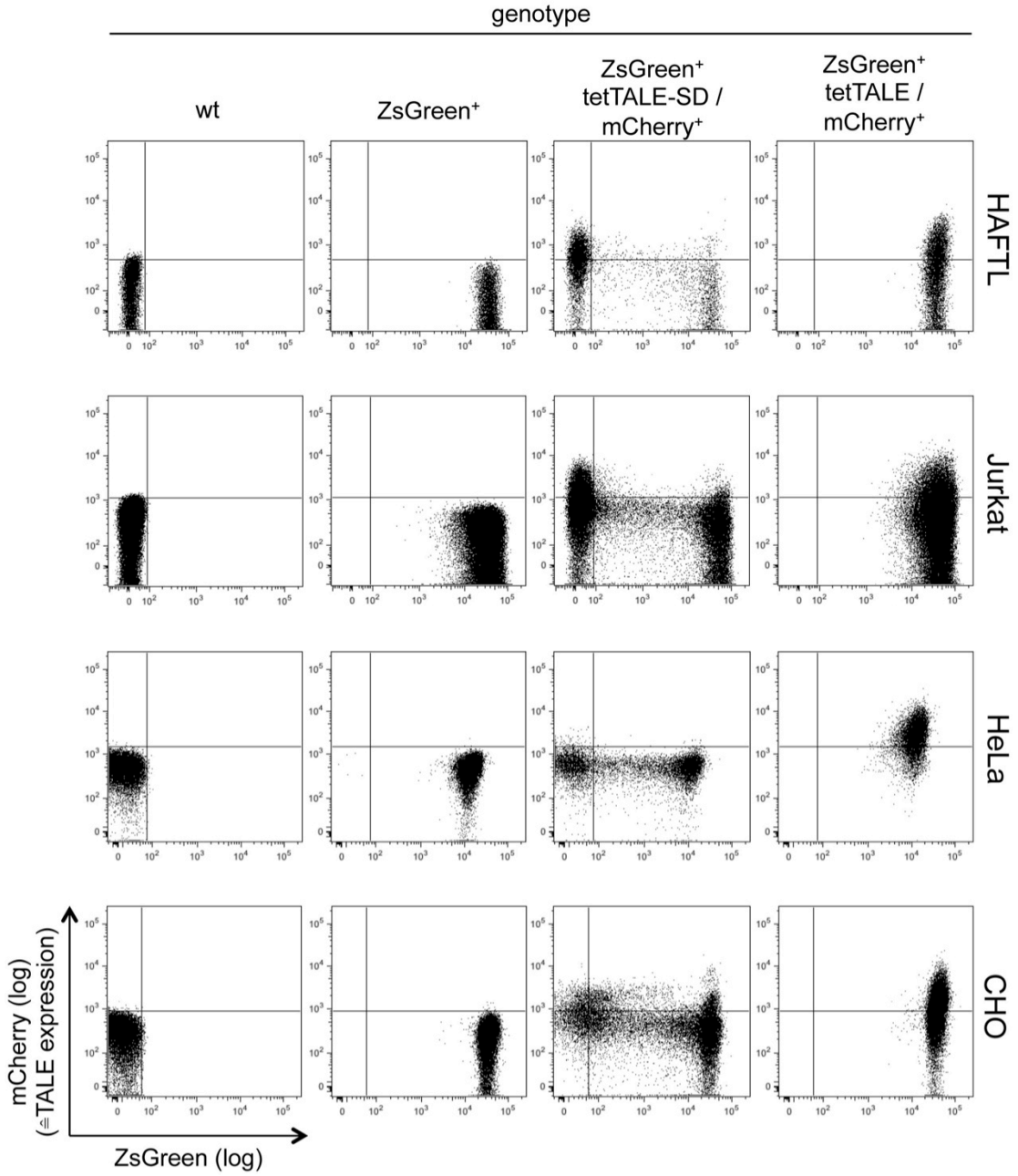

Figure 4.13 Stable *trans*-repression by tetTALE-SD
Four different cell lines carrying chromosomal copies of the tetEF-ZsGreen reporter were stably transfected with either tetTALE-SD or tetTALE and analyzed for ZsGreen and mCherry expression by FACS. Shown is the analysis of stable cell pools.
Partly published in Werner & Gossen, 2014 [108]

For the above-mentioned, reason HAFTL cells were chosen for clonal analysis. Clones were derived from the tetTALE-SD transfected pool by limited dilution and analyzed by microscopy, immunoblot and FACS for ZsGreen reporter and tetTALE-SD linked mCherry expression. All three methods consistently showed that high expression levels of tetTALE-SD correlated with substantial repression of the strong human EF1α promoter. Furthermore, it was shown that repression can be homogenous.

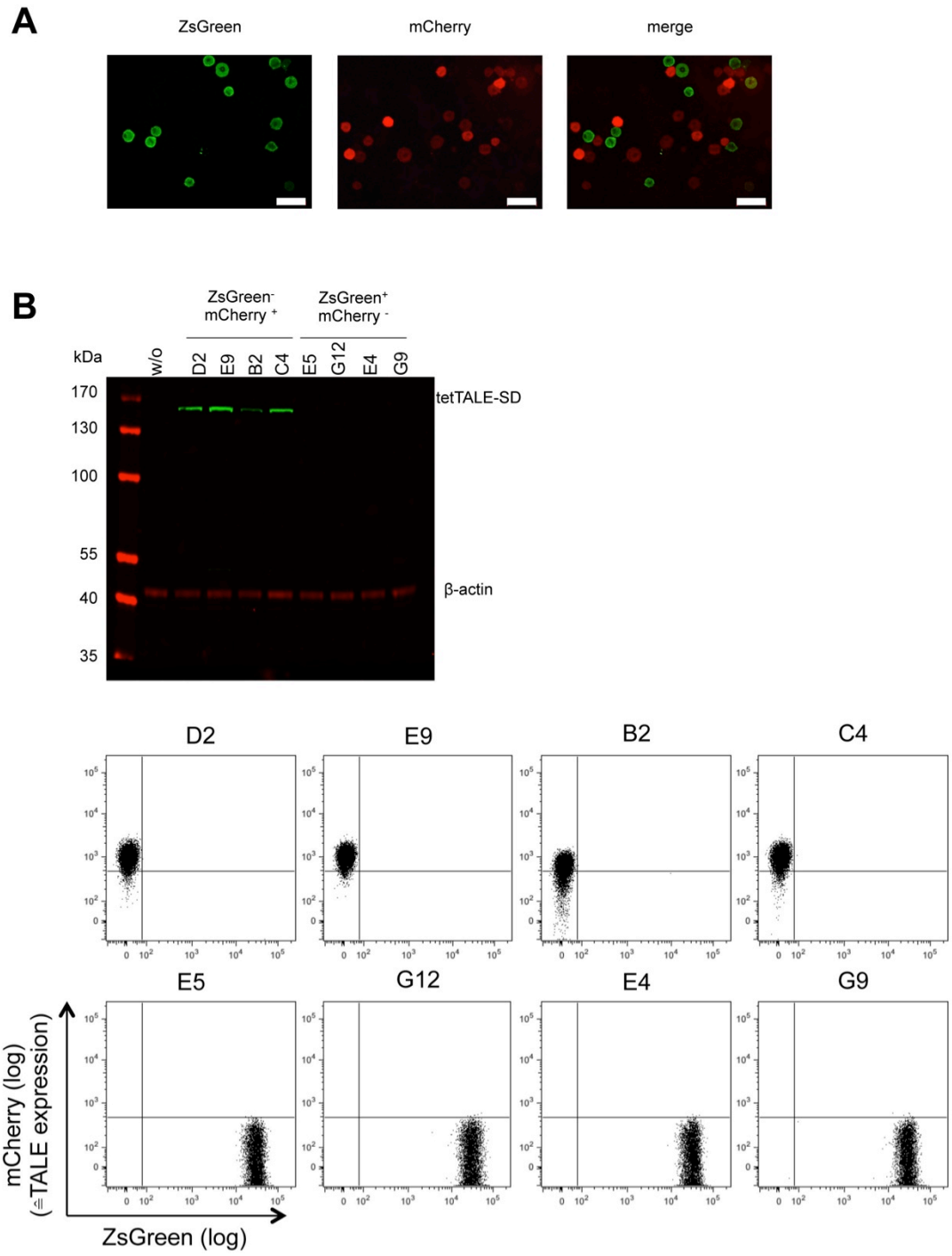

Figure 4.14 Clonal analysis of stable *trans*-repression by tetTALE-SD
(A) Microscopic picture of the HAFTL tetEF-ZsGreen cell pool after stable transfection with tetTALE-SD: ZsGreen (left), mCherry (middle), merge (right). Scale bar: 50 µm.
(B) Single clones isolated from the tetTALE-SD^{+} pool were analyzed by immunoblotting for tetTALE-SD expression levels with a α-HA antibody. β-actin levels served as a loading control. (C) FACS analysis of selected clones originating from HAFTL tetEF-ZsGreen cells after stable transfection with tetTALE-SD.
Partly published in Werner & Gossen, 2014 [108]

To demonstrate that the effective repression by an upstream TALE silencer is not exclusive for the hEF1α promoter used so far but an approach of general

applicability, the reporters tetRosa-ZsGreen and tetUbC-ZsGreen were constructed. These contained the human Rosa promoter or the human Ubiquitin C promoter, respectively (see Figure 4.15 A). Both reporters were stably transfected in HeLa cells. The resulting cell pools were then transfected with tetTALE or tetTALE-SD. As shown in Figure 4.15 B, both promoters were considerably repressed in the presence of tetTALE-SD. For the hRosa promoter, a distinct population with repression to background level was detected, whereas for the hUbC promoter the repression was more gradual. This substantiates the claim that tetTALE silencers can abrogate expression from strong mammalian promoters when located outside the core promoter.

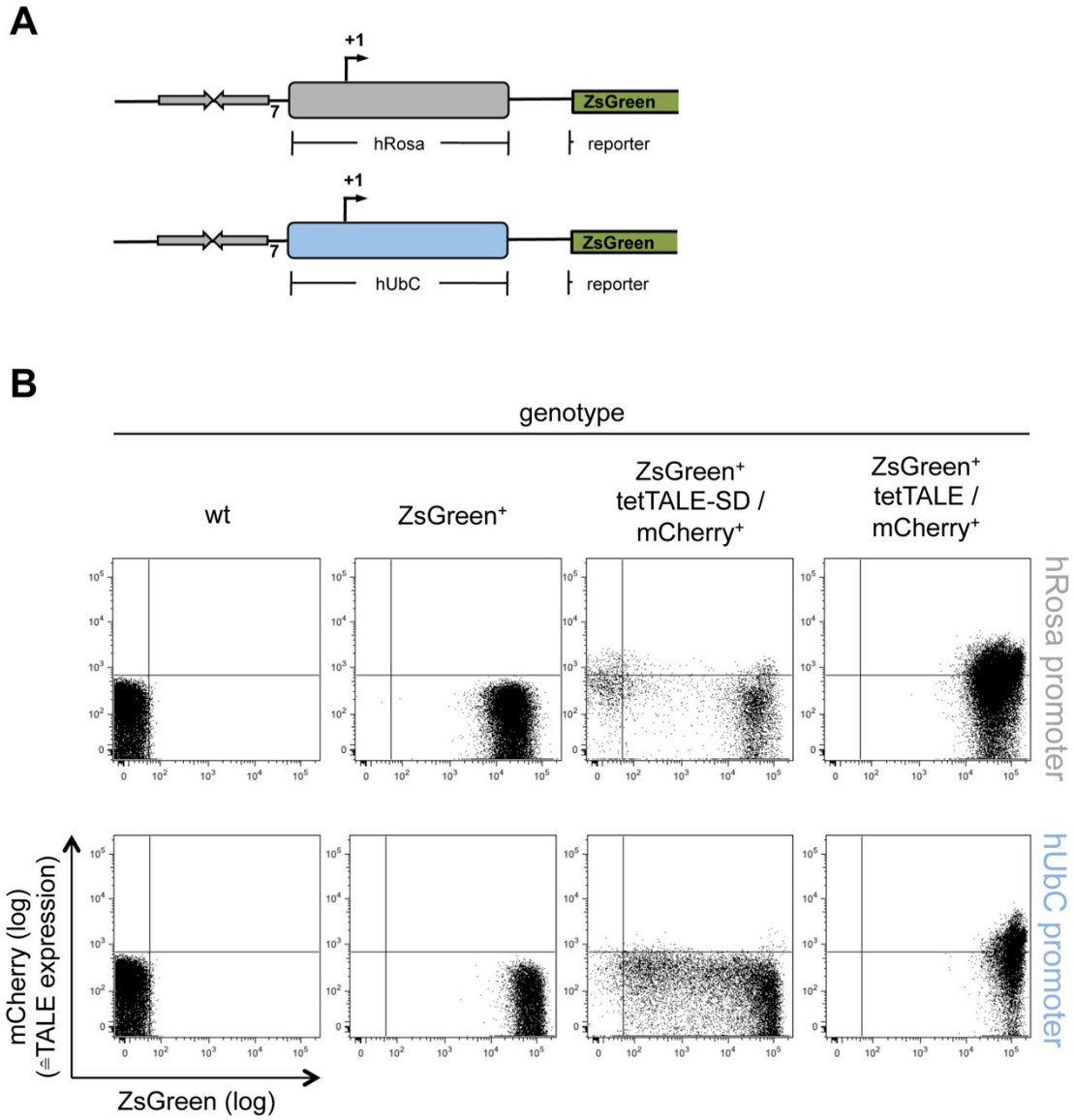

Figure 4.15 Stable *trans*-repression of additional mammalian promoters by tetTALE-SD
(A) Schematic picture of the tetRosa-ZsGreen and tetUbC-ZsGreen reporter constructs
(B) HeLA cell lines carrying chromosomal copies of either reporter were stably transfected with tetTALE-SD or tetTALE and analyzed for ZsGreen and mCherry expression by FACS. Shown is the analysis of stable cell pools.

4.3 The role of DNA methylation in tetTALE-SD-mediated repression

Transcription factors containing a KRAB domain are known to act on an epigenetic level [18,113]. In consequence the question was addressed if the strong repression observed with tetTALE-SD is in part due to epigenetic silencing. Considering that promoter regions are prone to activity changes depending on their methylation status [114], a methylation analysis for the region around the TSS of the chromosomal tetEF-ZsGreen reporter was performed. Genomic DNA was extracted and subjected to bisulfite conversion. Subsequently the region of interest, -191 to +111 relative to the TSS, was PCR-amplified. The PCR product was cloned in a TA cloning vector and analyzed by sequencing. For comparison, cells carrying the reporter only and single clones also expressing tetTALE-SD resulting in strong (clone C4) or no repression (clone E4) were analyzed. As shown in Figure 4.16, cells transfected with tetTALE-SD showed a more pronounced methylation of the promoter region than cells with the reporter alone. Moreover, clone C4, displaying strong repression of the reporter, showed a 25% increase in methylation compared to clone E4 where no repression was observed although the cells were transfected with tetTALE-SD. Despite this apparent influence of the KRAB domain on the methylation of the proximal promoter, there was a striking variability in the different samples for all groups analyzed (i.e plus and minus tetTALE-SD). One has to keep in mind that these stable tetEF-ZsGreen reporter cells were generated by transposition. As a consequence, multiple copies at various chromosomal loci have been integrated. As epigenetic modifications are particularly sensitive to position effects [115,116], each chromosomal reporter copy displays a different default methylation and is differently susceptible to changes caused by bound transcription factors.

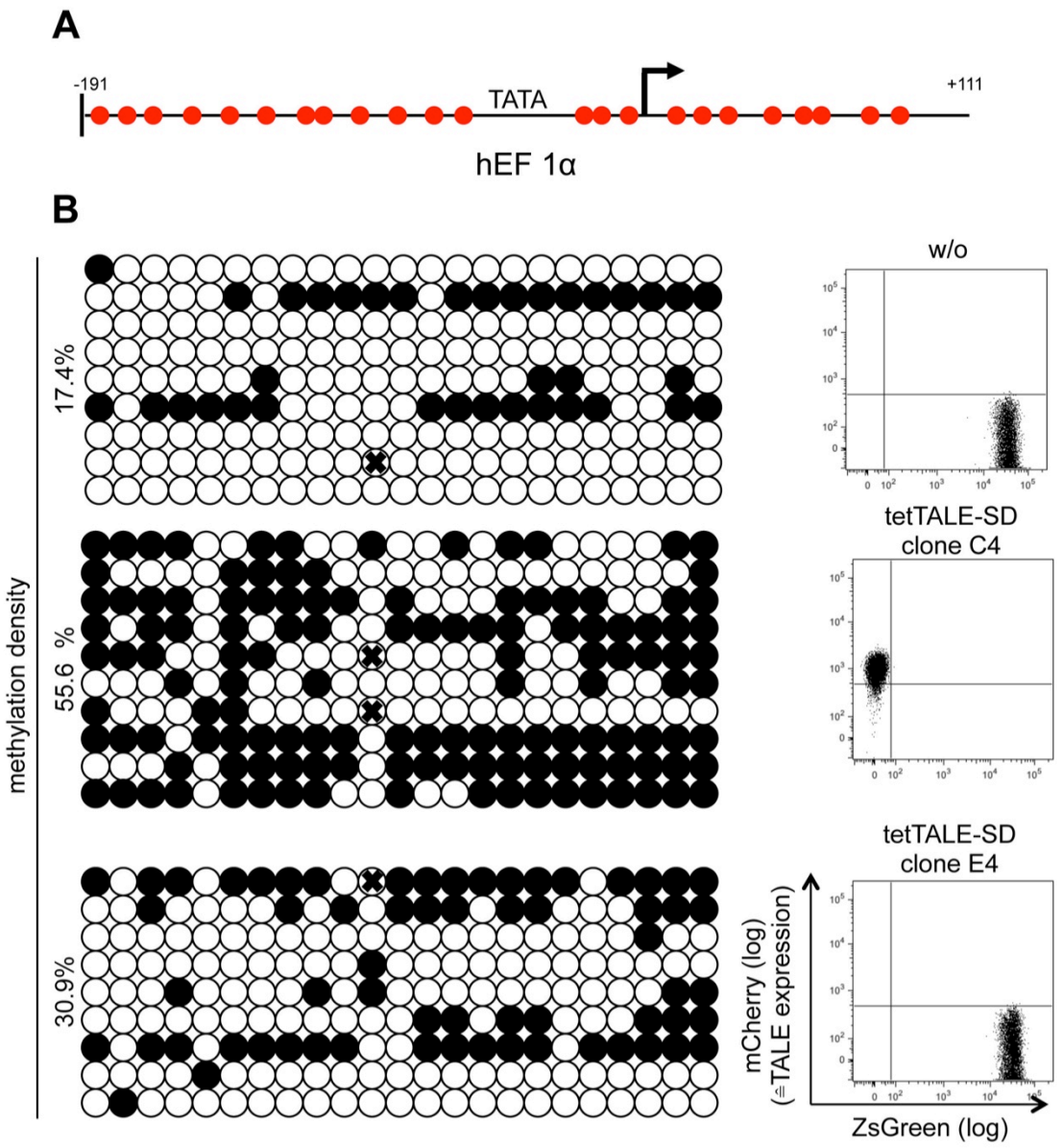

Figure 4.16 Methylation analysis of the hEF1α promoter
(A) Schematic representation of the analyzed region of the hEFα promoter position -191 to +111 (relative to the TSS) with each red dot representing a CpG dinucleotide.
(B) Methylation analysis of cells carrying the reporter only (w/o) and cell clones C4 and E4 additionally transfected with tetTALE-SD. Each row represents one isolate of the analyzed region of the hEF1α promoter, whereas each column represents a particular CpG position from different PCR products. Empty and filled dots represent unmethylated and methylated CpGs, respectively. Crosses mark sequencing ambiguities (left panel). FACS plots of the analyzed cells (right panel).

To overcome this limitation, cells with only a single chromosomal copy of the reporter were analyzed. Single copy integration was achieved by using a lentiviral construct (Q.V. Phan and M. Gossen, unpublished) comprising a hEFα driven d2EGFP reporter. The heptameric *tet*O sequence was introduced upstream of the promoter (see Figure 4.17 A). By transducing cells with a MOI of 0.1, single copy integration was favored. Single clones were selected for their high and homogeneous expression of the d2EGFP reporter. Following transfection with tetTALE-SD or

tetTALE, respectively, reporter expression was assessed by FACS. The experiment was carried out for two cell lines, namely HeLa and HAFTL. As expected from previous results, cells transfected with tetTALE-SD showed repression to background level, whereas reporter expression in tetTALE transfected cells was largely unaffected. However, the range of repression was smaller due to lower initial reporter expression, most likely resulting from the single copy integration (see Figure 4.17 B). Single clones derived from tetTALE-SD transfected HAFTL cells reinforced earlier results, showing that repression can be homogenous upon high expression of the silencer.

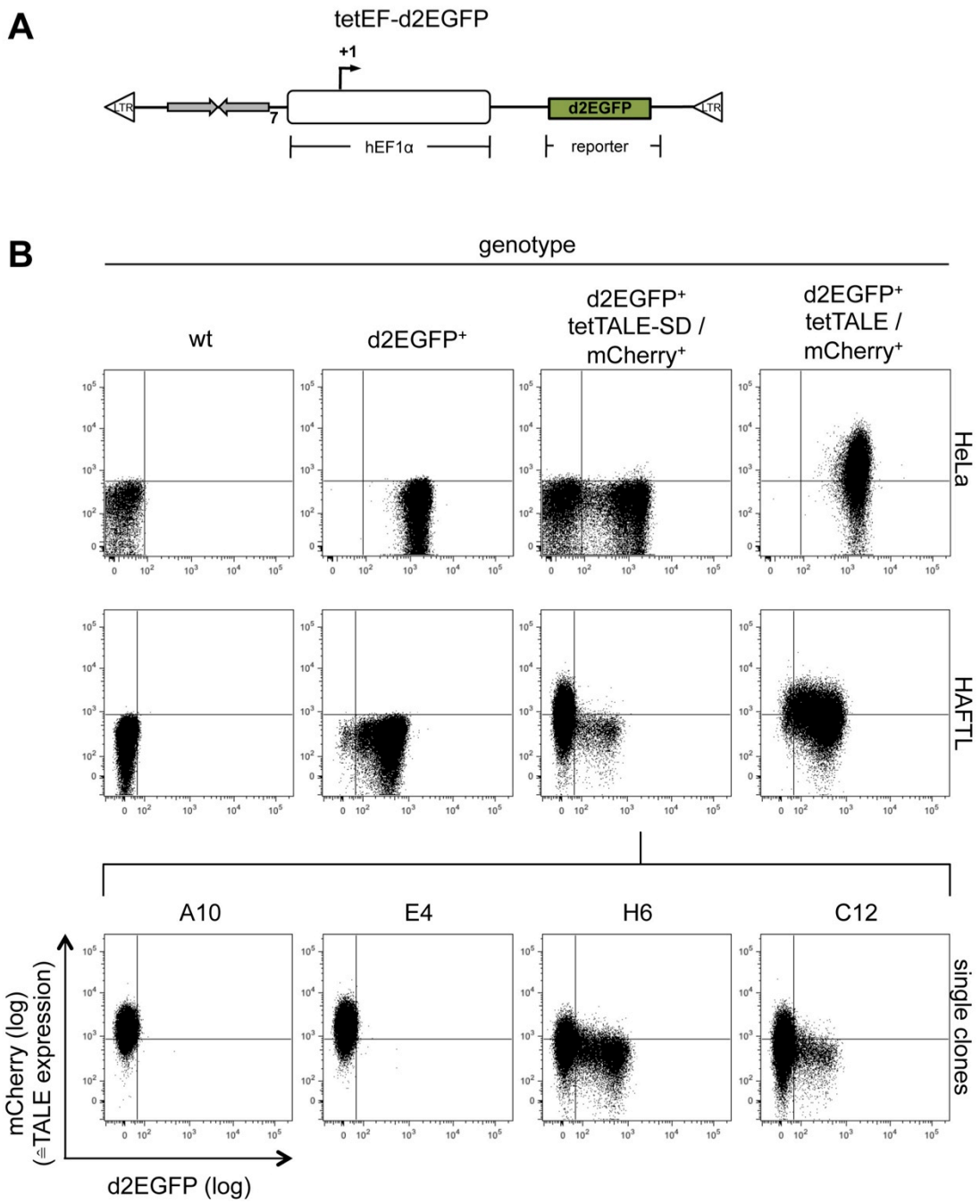

Figure 4.17.Stable *trans*-repression of a single copy reporter by tetTALE-SD
(A) A schematic picture of the lentiviral tetEF-d2EGFP reporter construct used for single copy integration (tetEF-d2EGFP). Lentiviral long terminal repeats (LTRs) are indicated
(B) Two different cell lines, carrying a single chromosomal copy of the tetEF-d2EGFP reporter, were stably transfected with either tetTALE-SD or tetTALE and analyzed for d2EGFP and mCherry expression by FACS. Single clones were selected from the HAFTL tetEF-d2EGFP /tetTALE-SD transfected pools and analyzed in the same way.

Thus, HAFTL clone A10 was chosen for the methylation analysis in comparison to cells before the introduction of tetTALE-SD. Contrary to expectations, no overall increase in methylation was detected after the addition of tetTALE-SD, despite the clear reduction in reporter expression (see Figure 4.18). However, one CpG located

199 bp downstream of the TSS was differentially methylated in that particular clone. To rule out that this result derived from a single clone is an artifact, it was decided to repeat the methylation analysis in cell pools to get information on average methylation changes caused by the KRAB domain and minimize the influence of integration loci.

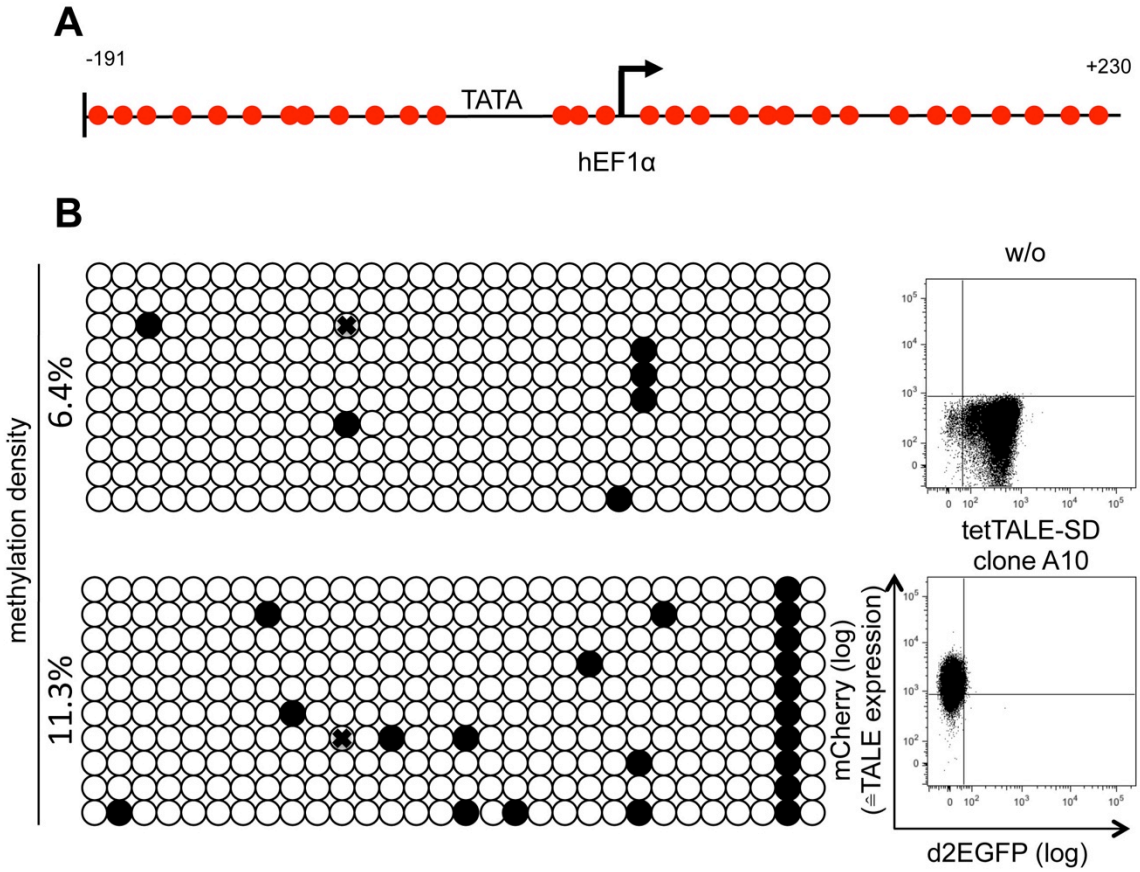

Figure 4.18 Methylation analysis of the hEF1α promoter after single copy integration
(A) Schematic representation of the analyzed region of the EF-promoter position -191 to +230 (relative to the TSS) with each red dot representing a CpG dinucleotide.
(B) Methylation analysis of cells carrying the reporter only (w/o) and cell clone A10 additionally transfected with tetTALE-SD. Each row represents one copy of the analyzed region of the hEF1α promoter, whereas each column represents a particular CpG position from different PCR products. Empty and filled dots represent unmethylated and methylated CpGs, respectively. Crosses mark sequencing ambiguities (left panel). FACS plots of the analyzed cells (right panel).

Therefore HAFTL cells, transduced with a MOI of 0.1 (about 65% d2EGFP$^+$), were sorted in a d2EGFP positive and negative fraction. Afterwards, the sorted cell fractions were analyzed for the ground state methylation pattern of the proximal hEF1α promoter, before the introduction of tetTALE-SD. However, the difference in reporter expression was not reflected in proximal promoter methylation, arguing against the concept that both aspects are mutually dependent in this particular setting (data not shown). At that point this part of the project was discontinued.

4.4 TAL effectors as roadblocks to transcription

Having established tetTALE-SD's ability to repress *in trans* when located upstream of the TSS, the question was addressed whether other modes of repression, omitting the silencing domain, would also work efficiently. Placing the target site in the transcribed region in marked distance to the promoter might enable the TALE to act as a roadblock for RNA polymerase II transcription. To test this assumption, a construct with a hEFα driven modified EYFP reporter was used. The open reading frame was interrupted by a synthetic intron (EYFPi; M. Gossen, unpublished) containing no, one or seven copies of the *tet*O. The *tet*O target was located 1627 bp downstream of the TSS (see Figure 4.19 A). HeLa cells, stably expressing tetTALE-SD or tetTALE, were transiently transfected with either one of the three reporter constructs. The expression of tetTALE before the introduction of the reporter was meant to favor repression. However, FACS analysis revealed that tetTALE was not able to counteract transcription elongation by RNAPII even with seven binding sites. Reduction of reporter expression was only observed in the presence of the silencing domain, arguing for a *trans*-repression effect rather than a steric roadblock. Yet it is noteworthy that with tetTALE-SD repression was enhanced when seven binding sites were present compared to only one (see Figure 4.19 B and C).

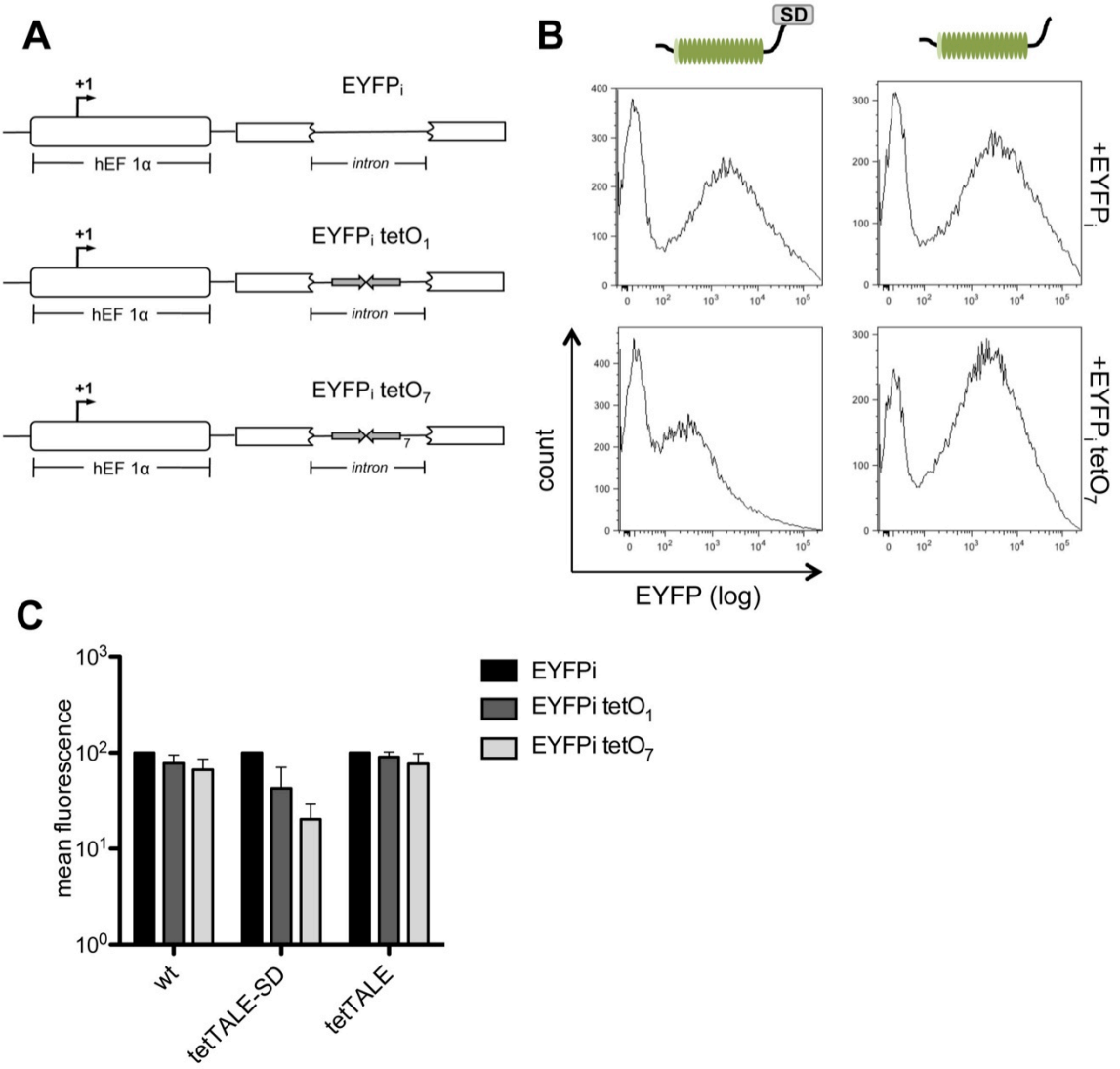

Figure 4.19 Transient elongation block
(A) Schematic presentation of reporter constructs. The hEF1α promoter was located upstream of a EYFPi reporter, containing an artificial intron. This intron was either unchanged (top) or carried one (middle) or seven (bottom) copies of the *tet*O.
(B) FACS analysis of HeLa cells stably expressing tetTALE or tetTALE-SD and transiently transfected with the reporter EYFPi or EYFPi $tetO_7$. Analysis was performed 48h after transfection.
(C) Quantification of the fluorescence signal of HeLa cells stably expressing tetTALE or tetTALE-SD and transiently transfected with the reporter EYFPi , EYFPi $tetO_1$ or EYFPi $tetO_7$. The signal intensity of cells transfected with the EYFPi reporter was set to 100. Shown are mean values of three independent transfections with standard deviation.

To enable the tetTALE to establish itself as a roadblock in an equilibrium situation, both tetTALE and the reporter constructs were stably integrated in HeLa cells. First single clones were picked with regard to high reporter expression, followed by stable integration of either tetTALE or tetTALE-SD. In addition, a tetTALE targeting the opposite strand of the *tet*O ($tetTALE_{rev}$) and tetR without silencing domain were tested in parallel. However, tetTALE was not able to suppress reporter expression, independent on the number of binding sites ($tetO_1$ or $tetO_7$) and the target strand

(see Figure 4.20 middle and right panel). The same holds true for tetR. Again, the only distinct reduction or reporter expression was observed when tetTALE-SD was bound to the heptameric *tet*O target (see Figure 4.20 right panel).

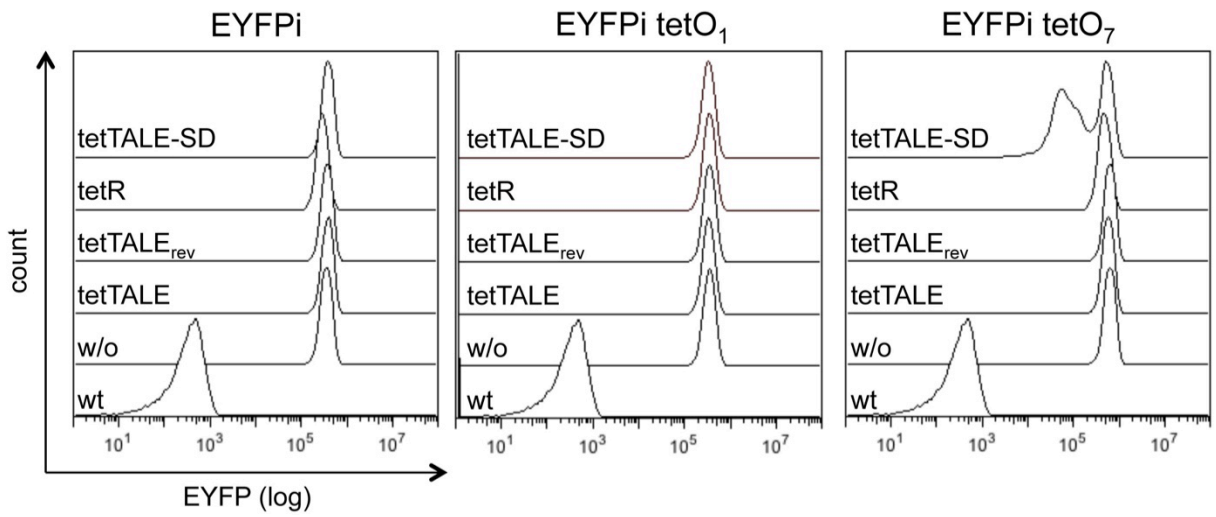

Figure 4.20 Stable elongation block
HeLa cells were stably transfected with the EYFPi, EYFPt $tetO_1$ or EYFPi $tetO_7$ reporter cassette and single clones were selected. Afterwards tetTALE, $tetTALE_{rev}$, tetTALE-SD or tetR were stably transfected, in a selected $EYFP^+$ clone and EYFP expression was assessed by FACS analysis.
Partly published in Werner & Gossen, 2014 [108]

Apparently tetTALE binding does not counteract strand elongation by RNAPII, independent of target frequency or DNA strand targeted.

4.5 Inducible TALE function

Conditional transcriptional regulation of endogenous genes would broaden the range of possible applications. Therefore, the next step was to establish such a system for the presented tetTALE. The logical choice for conveying conditionality to TALEs would be the Tet system [117,118]. However, this is not possible for the experimental setting presented here, as tetTALEs share identical binding specificity with the tetR-based transcription factors used in all tetracycline-controlled transcription systems. Hence, two other systems were tested, namely the iDimerize system and the retinoid x/ecdysone gene switch. Both were previously established for conditional gene control relying on small molecules [94,119].

4.5.1 iDimerize System

The iDimerize system (Clontech) is based on the natural eukaryotic heterodimerizer rapamycin and its binding partners, the human proteins FKBP12 and FRAP. FKBP acts as a cytoplasmic receptor for rapamycin, whereas FRAP is a phosphatidylinositol 3-kinase homolog [120].

The system has three main components. The first component is a DNA-binding domain fused to three FKBP domains (also termed DmrA domain) and a nuclear localisation sequence enabling nuclear transport. The second component is a transcriptional activator fused to a portion of FRAP (also termed DmrC domain). Hereafter those two elements are referred to as the activation components of the system. Thirdly, an analog to rapamycin (also termed A/C Heterodimerizer), which does no longer interact with its natural binding partners, is used to induce proximity of the DNA-binding domain and the activation domain via heterodimerization of DmrA and DmrC. The NLS ensures that both components are localized to the nucleus and perform their function as an activating transcription factor. In the absence of the heterodimerizer, only the DNA-binding domain is located in the nucleus and does not cause transcriptional activation (see Figure 4.21).

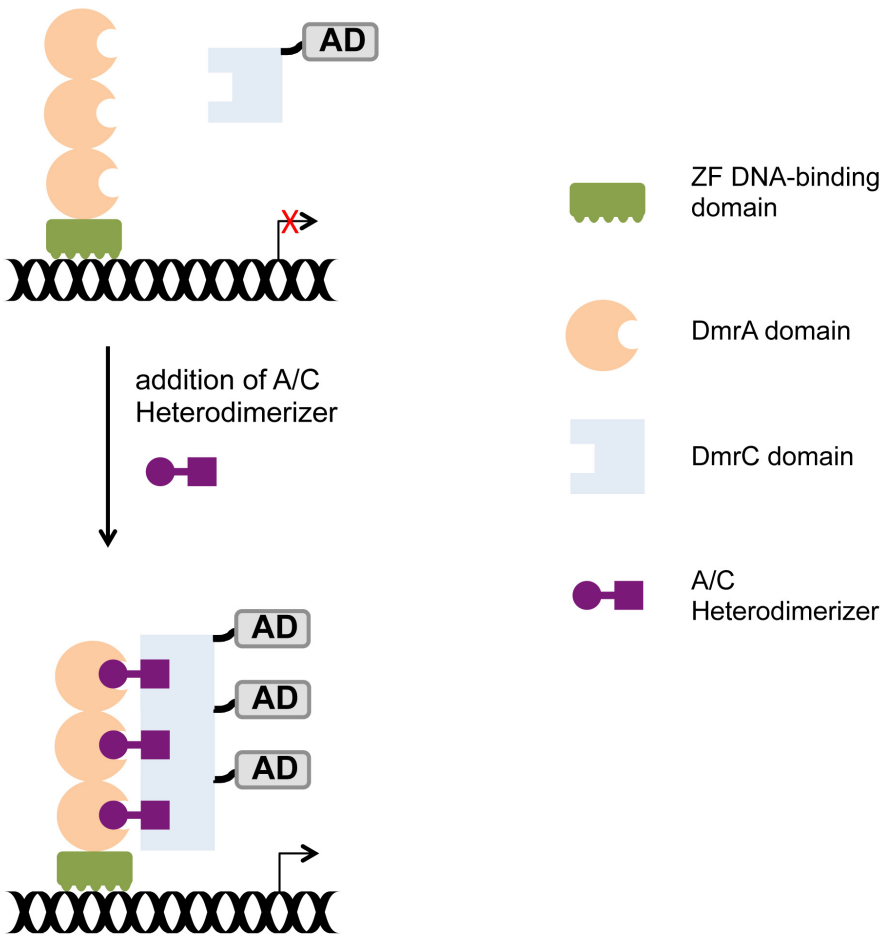

Figure 4.21 Functional principle of the iDimerize system
A DNA-binding domain fused to three copies of DmrA and a transcriptional activation domain (AD) fused to a DmrC domain constitute the activation components of the system. The presence of the A/C Heterodimerizer results in the induced proximity of both activation components at a promoter and initiates gene expression. The nomenclature used in this scheme is that from the commercial supplier of the regulatory system, Clontech.
Adapted from Pollock *et al.* [121]

The reporter construct used for conditional expression contained 12 binding sites for the zinc finger, used as a DNA-binding domain, upstream of a minimal IL2 promoter. In the following experiment tetTALE-SD was put under the control of this promoter to make its expression dependent on the presence of the heterodimerizer. The inducible tetTALE-SD and the expression constructs for the two activating components were stably transfected in HAFTL tetEF-ZsGreen cells, used before for repression experiments. It was expected that in the presence of the A/C Heterodimerizer the expression of tetTALE-SD is initiated, ultimately resulting in a repression of the ZsGreen reporter. Without addition of the heterodimerizer, tetTALE-SD should not be transcribed and the expression of the ZsGreen reporter should remain unaffected (see Figure 4.22 A). As shown in Figure 4.22 B, the repression

achieved in the presence of the heterodimerizer (i.e. tetTALE-SD expression) was only marginal. These results point at a rather low efficiency of the iDimerize system, at least in this particular experimental setting. Therefore, efforts were focused on the alternative conditional TALE expression system, namely the retinoid X/ ecdysone receptor gene switch [122].

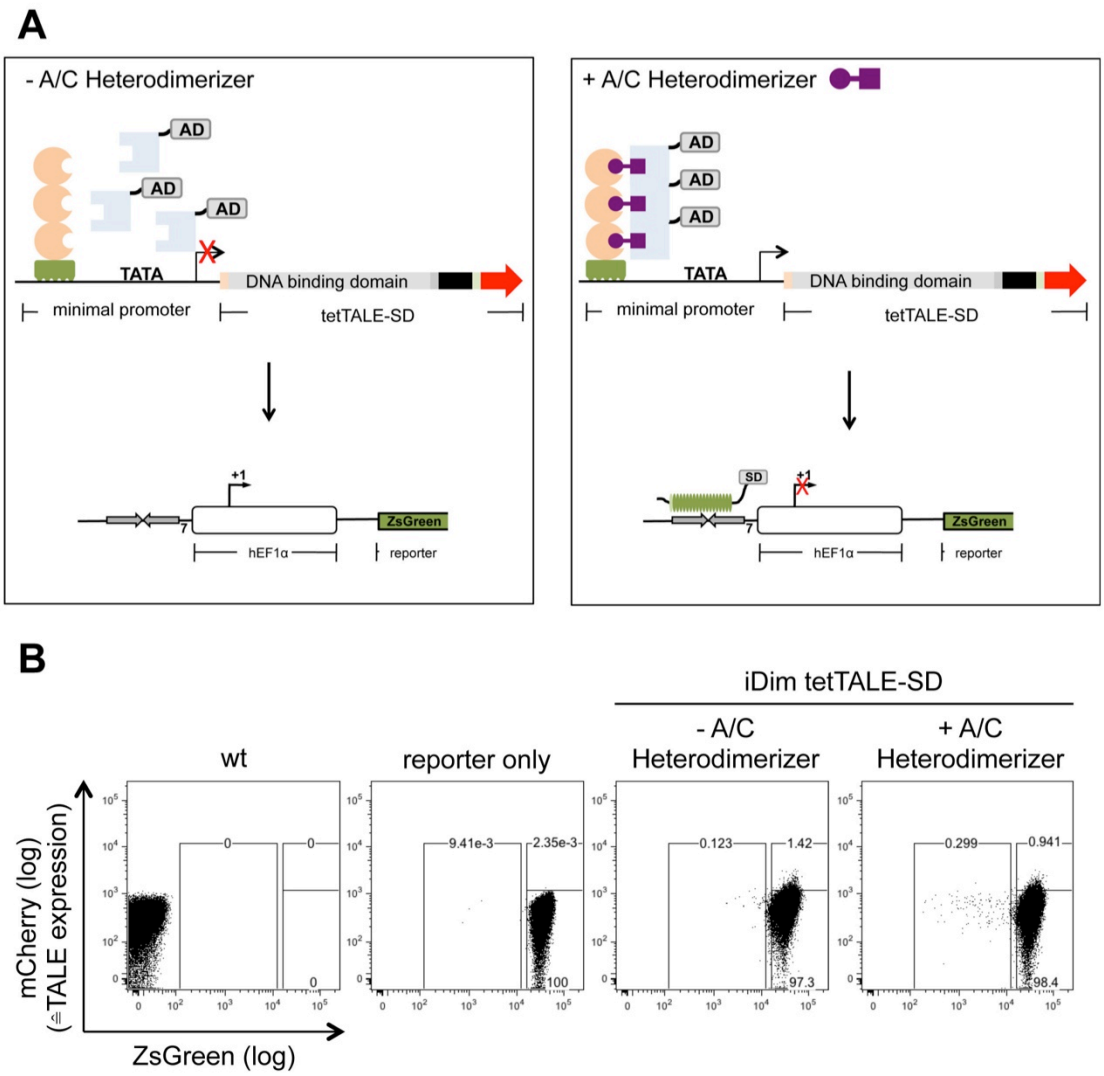

Figure 4.22 Heterodimerizer-induced tetTALE-SD-mediated repression
(A) Experimental design. TetTALE-SD expression was put under the control of a heterodimerizer inducible promoter (iDim tetTALE-SD). This construct, as well as the expression constructs for the activating components of the iDimerize system (DmrC/DmrA), were stably transfected in HAFTL tetEF-ZsGreen cells. In the absence of the heterodimerizer, tetTALE-SD was not expressed and the ZsGreen reporter was unaffected (left panel). Upon the addition of A/C Heterodimerizer transcription of tetTALE-SD was induced, resulting in a repression of ZsGreen reporter expression (right panel).
(B) FACS analysis of unmodified HAFTL cells (wt), HAFTL tetEF-ZsGreen cells (reporter only) and cells stably carrying the inducible tetTALE-SD cassette and both activating components in the absence (-A/C Heterodimerizer) or presence (+A/C Heterodimerizer) of the heterodimerizer. Shown is the analysis of stable cell pools.

4.5.2 Retinoid X/ ecdysone gene switch

This system represents a single chain genetic switch. It is comprised of a DNA-binding domain, a RXE unit and an effector domain. The RXE unit consists of a retinoid X receptor-α is linked to an ecdysone receptor. Upon the addition of the insect hormone ponasterone A (PonA), the retinoid X receptor-α and the ecdysone receptor undergo intramolecular rearrangement, resulting in the formation of a functional transcription factor. The main advantage of the system is that only one component needs to be delivered. It was shown that in this setting TALEs can be used as DNA-binding domains and transiently activate gene expression in an inducible fashion [94]. In this thesis, the RXE complex was fused between the C-terminal part of tetTALE and the N-terminal end of an effector domain, like the VP64 activation domain, as described by Mercer *et al*. [94]. A scheme of the conditional tetTALE system is given in Figure 4.23.

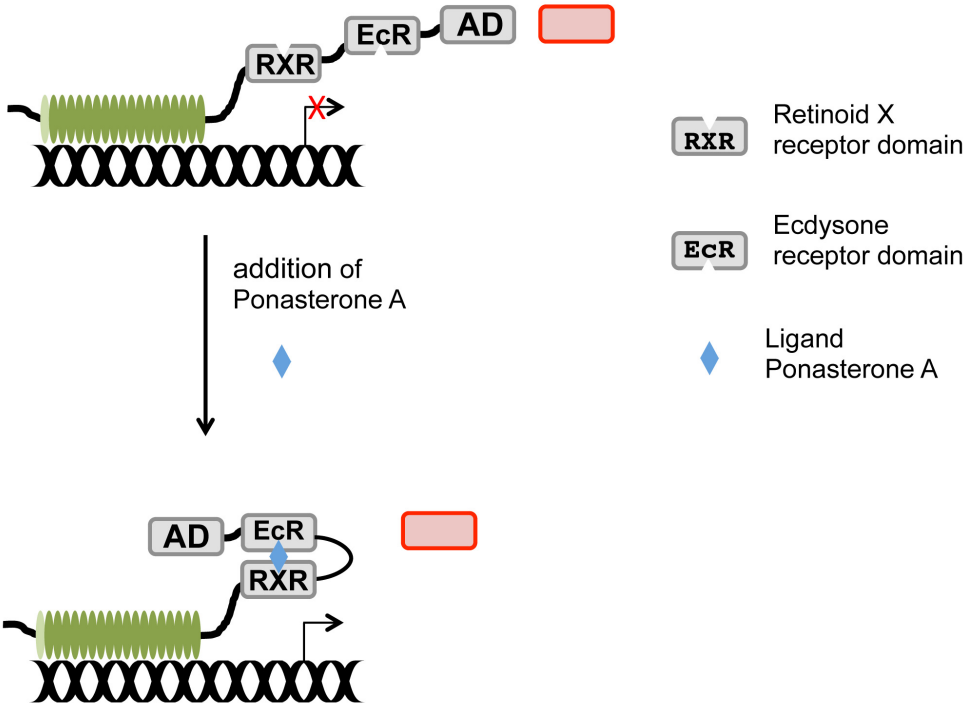

Figure 4.23 Schematic representation of the functionality of the retinoid x/ecdyson gene switch
The retinoid X receptor α (RXR) and the ecdysone receptor (EcR) are located between the tetTALE DNA-binding domain and the VP64 activation domain (AD). A mCherry fluorescent marker is linked via a T2A site. Without the addition of the ligand ponasterone A, the tetTALE-RXE-AD can bind to the target sequence (upper panel) but without exerting transcriptional control. The addition of PonA causes an intramolecular reorganization, resulting in a functional transcriptional activator and the expression of the reporter gene (lower panel).
Adapted from Mercer *et al*. [94]

As a first step, the functionality of tetTALE-RXE-AD was tested. Therefore, the previously described X1/6 cells [33], carrying chromosomal copies of P_{tet7} luc, were transfected with tetTALE-RXE-AD with or without the addition of PonA. Constitutively active tetTALE-AD was used for comparison. Without the ligand, only marginal activity of the luciferase reporter was observed compared to mock transfected cells (w/o). Addition of PonA resulted in a 98-fold increase of reporter expression, slightly exceeding even the activation achieved by tetTALE-AD (see Figure 4.24). This clearly demonstrates the functionality and broad range of inducibility of the retinoid X/ ecdysone receptor gene switch in combination with tetTALE-AD, rendering the system suitable for further investigation.

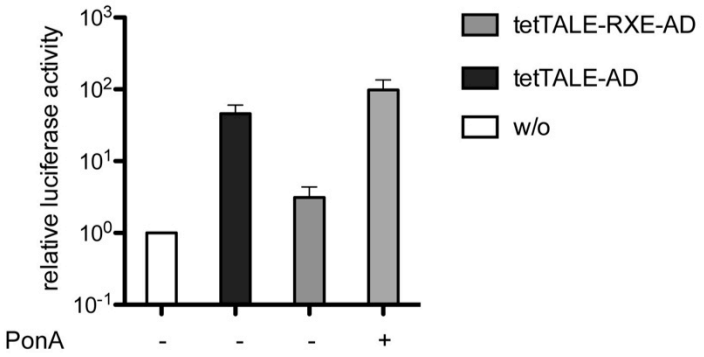

Figure 4.24 Activation of a chromosomal target with tetTALE-RXE-AD
X1/6 cells, carrying P_{tet7} luc stably integrated in the chromosome, were transfected with either a tetTALE-RXE-AD or av tetTALE-AD expression construct. The transfection was performed with or without the addition of 10 µM ponasterone A (PonA). A *Renilla* luciferase expression construct was included for internal standardization. P_{tet7} activity in mock transfected cells was set to 1. Shown are mean values of three independent transfections with standard deviation.

Next, the kinetics of PonA-induced activation was addressed. This time tetTALE-RXE-AD and tetTALE-AD were stably integrated in X1/6 cells [33]. Cells were then cultivated for two, four and seven days plus or minus PonA, and luciferase reporter activity was determined. As shown in Figure 4.25, tetTALE-AD-mediated activation was on average 300-fold, constant over time and expectably unaffected by the presence of PonA. As expected, tetTALE-RXE-AD-induced activation was highly dependent on PonA, already showing high activation levels on day 2 and reaching a maximum of 500-fold activation on day 7. At all time points, the background activity of tetTALE-RXE-AD minus PonA was relatively low and constant. This proves that tetTALE-RXE-AD activation works under stable conditions with fast kinetics and moderate background.

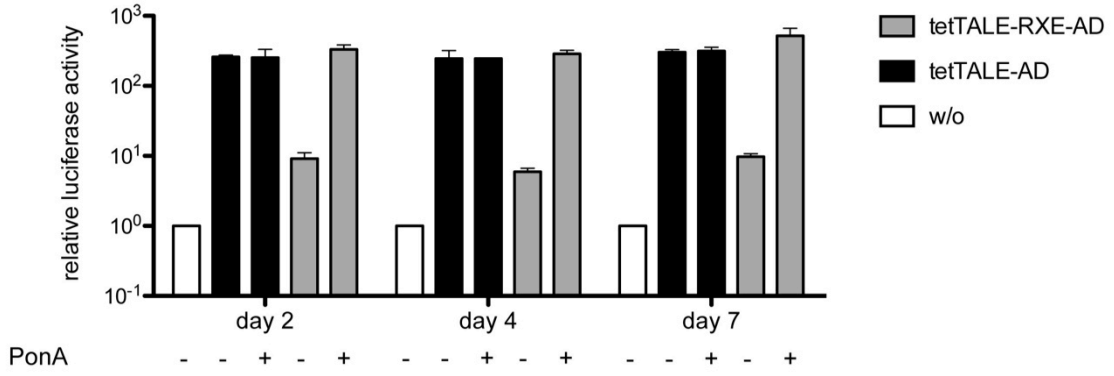

Figure 4.25 Time course of stable activation of a chromosomal target by tetTALE-RXE-AD
X1/6 cells, carrying the P_{tet7} luc reporter stably integrated in the chromosome, were stably transfected with either a tetTALE-RXE-AD or a tetTALE-AD expression construct. The cells were then cultured with or without the addition of 10 µM PonA for two, four and seven days. Luciferase reporter activity was measured and normalized to protein content. Reporter activity in untransfected X1/6 cells (w/o) served as a reference for all time points and was set to 1. Shown are mean values of three independent stable transfections with standard deviation.

Having shown that tetTALE-RXE-AD works with good inducibility and low background, the next step was to utilize that system for tetTALE-SD-mediated repression. Therefore, the RXE complex was integrated in the tetTALE-SD and tetTALE constructs, resulting in tetTALE-RXE-SD and tetTALE-RXE. Both constructs were stably transfected in HAFTL tetEF-ZsGreen cells. After addition of PonA, the change in ZsGreen expression in cell pools was analyzed at three time points to assess the kinetics of repression. In line with the previous experiments, there was no change in reporter expression in tetTALE-RXE transfected cells after the addition of PonA due to the lack of a silencing domain. By contrast, there was a clear reduction in ZsGreen reporter expression in tetTALE-RXE-SD transfected cells cultured in the presence of PonA, reaching a maximum on day four (Figure 4.26). The reduction ranged from only intermediate decrease (Figure 4.26 middle gate) to total abrogation of expression (Figure 4.26 left gate). The majority of cells showed an intermediate repression of more than 10-fold compared to untransfected cells, whereas in a maximum of 7.7 % of the cells the ZsGreen repression was to background level (1100-fold). However, about half of the population did not show a shift in reporter expression and cells cultured in the absence of PonA showed a minor shift to the left, indicating a background activity of tetTALE-RXE-SD.

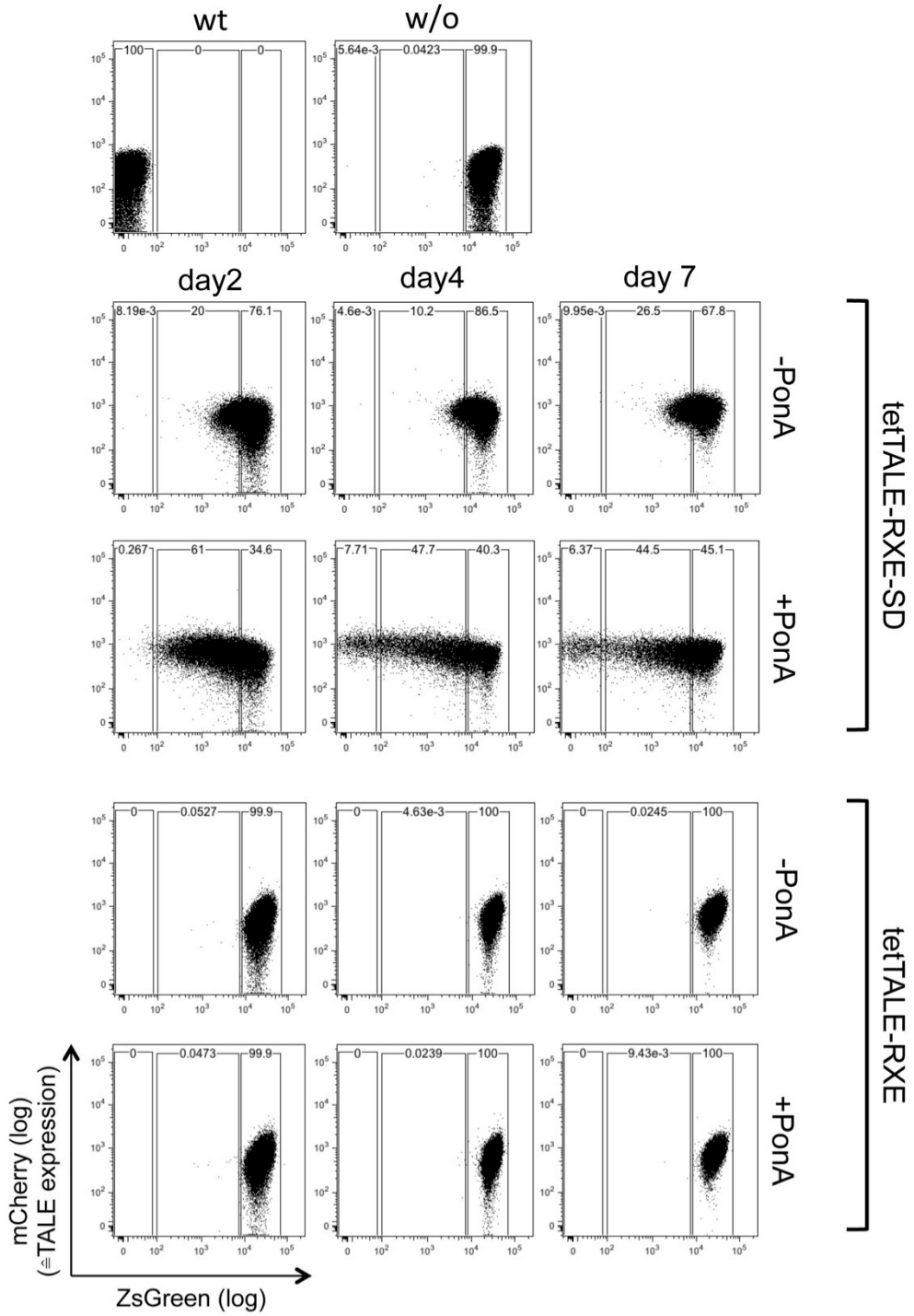

Figure 4.26 Time course of stable repression of a chromosomal target by tetTALE-RXE-SD HAFTL tetEF-ZsGreen cells were stably transfected with either tetTALE-RXE-SD containing a KRAB silencing domain or tetTALE-RXE with no effector domain. Cells were then cultured in the presence or absence of 10 µM PonA and ZsGreen reporter expression was monitored via FACS on day 2, 4 and 7. HAFTL wildtype cells (wt) and untransfected HAFTL tetEF-ZsGreen cells (w/o) served as a control. Shown is the analysis of stable cell pools.

The observed heterogeneity led to the decision to select single clones of tetTALE-RXE-SD transfected cells, with a minimum of background activity and maximum inducibility of repression. Three representative clones are shown in Figure 4.27,

demonstrating that, as expected from pool analysis, the responsiveness to PonA differed clearly between the clones. No ligand-independent repression was observed, but still a notable fraction of the cells showed unchanged reporter expression in the presence of PonA. Clone A9 was chosen for subsequent experiments.

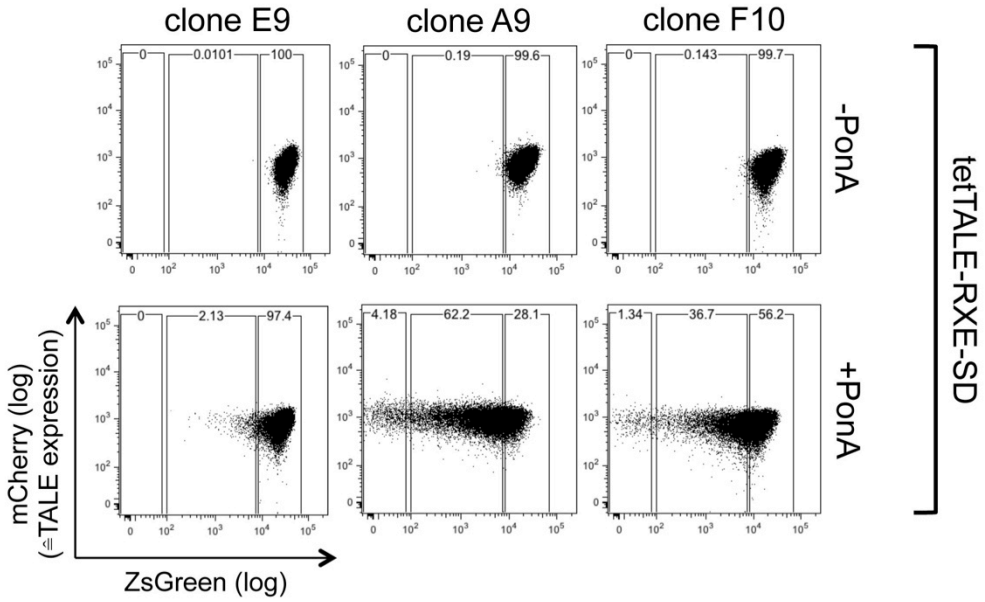

Figure 4.27 PonA responsiveness of tetTALE-RXE-SD transfected HAFTL tetEF-ZsGreen cells Single clones were isolated from HAFTL tetEF-ZsGreen cells stably transfected with tetTALE-RXE-SD. Cells were cultured for seven days in the presence or absence of 10 µM PonA and ZsGreen expression was determined by FACS analysis. Shown is the analysis of three representative clones.

The retinoid X/ ecdysone receptor gene switch allows not only an ON or OFF switch but has been shown to be adjustable, depending on the administered PonA concentration [94]. To test the tuneability of the tetTALE-RXE-SD-mediated repression, clone A9 was cultured with different PonA concentrations ranging from 0,1 µM up to 10 µM for four days. FACS analysis of ZsGreen reporter expression evidenced that there was indeed a clear correlation between increasing PonA concentrations and an increasing repression, manifested in a decrease of mean fluorescence. This is in accordance to previous observations of PonA-dependent transcription factor action [94].

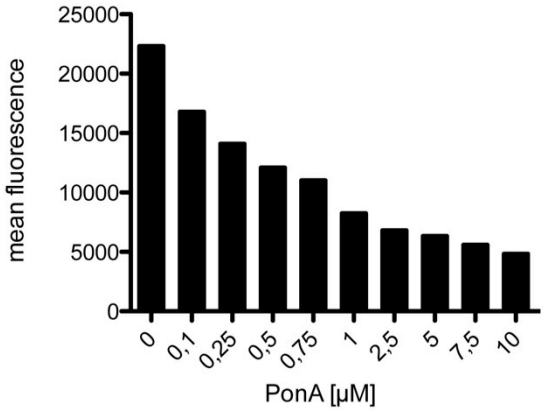

Figure 4.28 Dependence of tetTALE-RXE-SD-mediated repression on the PonA concentration
The single clone A9 of HAFTL tetEF-ZsGreen cells stably transfected with tetTALE-RXE-SD was cultured with different concentrations of PonA for 4 days. The ZsGreen expression level was analyzed by FACS measurement.

The question arose if a reversal of the repression could be achieved by a removal of PonA. As starting cells, a homogeneously repressed population was needed. Therefore, clone A9 was cultured for 17 days with 10 µM PonA to ensure a steady state of repression. Cells with total abrogation of ZsGreen expression were isolated via FACS sort (see Figure 4.29 day, 0) and continuously cultured in the presence of PonA, i.e. continuous reporter repression. However, FACS analysis on day 7 and day 12 after the sort revealed that the sorted population was unstable and returned to the initial ZsGreen expression pattern. When PonA was removed on day 12 from a fraction of the cells, ZsGreen completely recovered to its initial expression state. Seven days after PonA removal the cells showed a high reporter expression level resembling that of the parental HAFTL tetEF-ZsGreen cells. This demonstrates that the repression is completely reversible (see Figure 4.29).

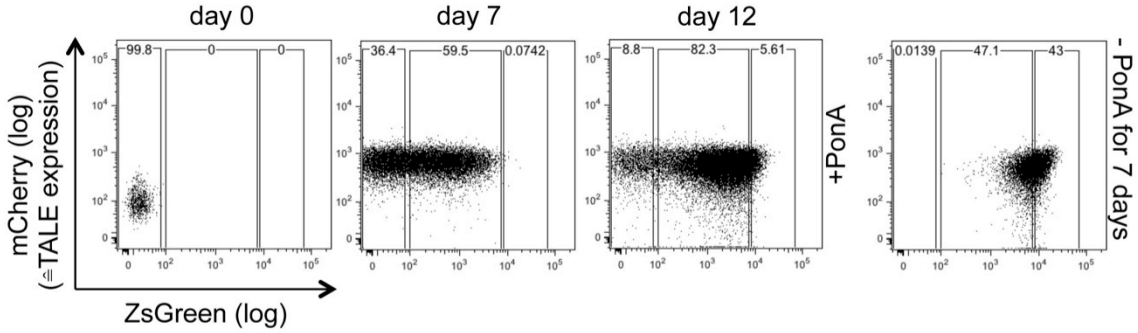

Figure 4.29 Stability of cells sorted for complete repression mediated by tetTALE-RXE-SD
The single clone A9 of HAFTL tetEF-ZsGreen cells, stably transfected with tetTALE-RXE-SD, was cultured for 17 days with 10µM PonA. Cells with complete ZsGreen reporter repression were collected via FACS sorting (day 0) and subsequently cultured in the presence of PonA. Reporter levels were assessed on day 7 and 12 after the sort. From day 12 on PonA was removed for 7 days from the culture and ZsGreen expression was again measured via FACS analysis.

One possible explanation for the instability of repression mediated by tetTALE-RXE-SD is its heterogeneous expression. The expression of tetTALE-RXE-SD in the previous experiments was driven by the CMV promoter that sometimes caused irregularities in tetTALE-SD expression levels (data not shown). Hence, it was decided to use the human EF1α to drive the tetTALE-RXE-SD cassette in expectation of a more stable expression. HAFTL tetEF-ZsGreen cells were again stably transfected, this time with the EF tetTALE-RXE-SD. Single clones were selected for the absence of ligand-independent repression and high reduction of ZsGreen reporter after the addition of PonA. Corresponding to the earlier findings, the responsiveness to PonA differed among the clones (see Figure 4.30). However, the degree of repression clearly lagged behind results obtained with CMV promoter driven tetTALE-RXE-SD. Nevertheless clone B8 was chosen for further experiments.

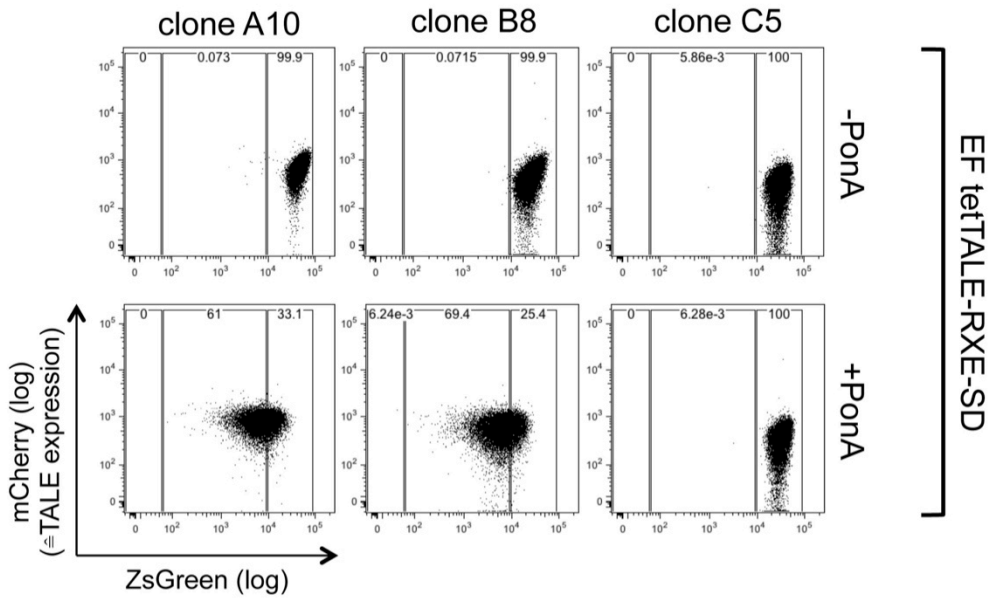

Figure 4.30 PonA responsiveness of EF tetTALE-RXE-SD transfected HAFTL tetEF-ZsGreen cells
Single clones were isolated from HAFTL tetEF-ZsGreen cells, stably transfected with EF tetTALE-RXE-SD. Cells were cultured for four days in the presence or absence of 10 µM PonA and ZsGreen expression was determined by FACS analysis. Shown is the analysis of three representative clones.

Clone B8 was kept in culture with 10µM PonA for 18 days and successively sorted twice to obtain a stable ZsGreen negative population, i.e. cells with complete repression. Follow-up FACS analysis, at three time points after the second sort, revealed an increased but still insufficient stability of repression. Nonetheless, 34% of the cells remain ZsGreen negative on day 21 after the sort, compared to only 8.8% with CMV tetTALE-RXE-SD on day 12. This provides evidence that the combination

of two subsequent sorts and the usage of the hEF1α promoter helped improving the stability of the repressed population.

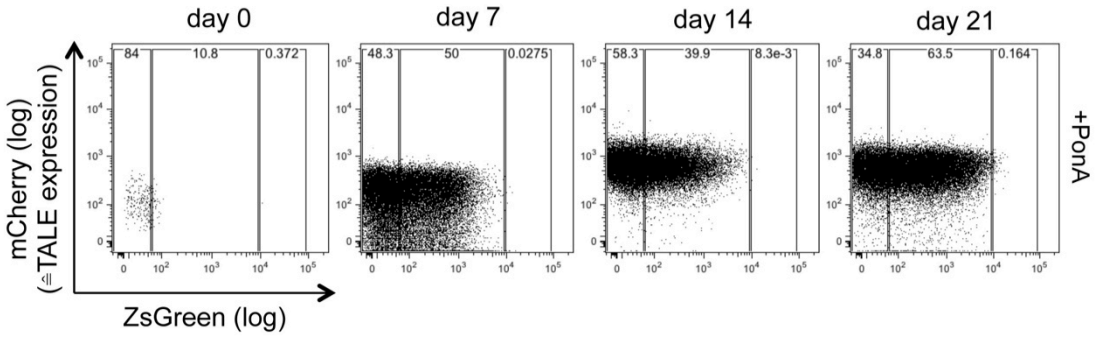

Figure 4.31 Stability of cells sorted for complete repression mediated by EF tetTALE-RXE-SD
Clone B8 of HAFTL tetEF-ZsGreen cells, stably transfected with EF tetTALE-RXE-SD, was cultured for 18 days with 10µM PonA. Cells with complete ZsGreen reporter repression were collected via FACS sorting twice (day 0 represent cells after the second sort) and subsequently cultured in the presence of PonA. Reporter levels were assessed on day 7, 14 and 21 after the sort.

On day 7 after the sorting of clone B8, a fraction of the cells was cultured without PonA for 7 days, followed by the addition of PonA for another 7 days. FACS analysis of ZsGreen reporter expression illustrates that repression was almost completely reversible by removing PonA for 7 days. Reinducing tetTALE-RXE-SD-mediated repression by adding PonA also engenders clear reduction in ZsGreen expression in almost 100% of the cells, but did not result in repression levels comparable to the initial state (see Figure 4.32).

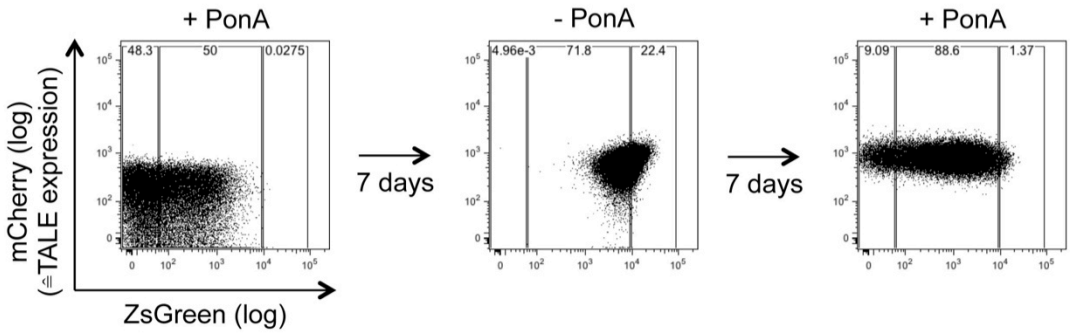

Figure 4.32 Reversibility of EF tetTALE-RXE-SD-mediated repression
HAFTL tetEF-ZsGreen EF tetTALE-RXE-SD clone B8 was cultured with 10 µM PonA and sorted for total abrogation of ZsGreen signal. Seven days after the sort (left) PonA was removed from the culture for seven days (middle), followed by seven days of culture in the presence of 10 µM PonA (right). ZsGreen reporter expression was analyzed by FACS.

The retinoid X/ecdysone gene switch had shown its principal functionality with good dose-dependent and reversible repression by tetTALE-RXE-SD. Despite this proof of principle, the stability of repression in a sorted cell population posed a challenge. To

exclude intrinsic cell line specific factors it was decided to introduce the system in three additional cell lines, namely HeLa, CHO and HEK293 TN cells. To this end, stable tetEF-ZsGreen clones were created for all three cell lines and selected for high and homogenous reporter expression. Afterwards, EF tetTALE-RXE-SD was stably introduced and the cells were cultured for 7 days with or without PonA. FACS analysis showed that PonA-induced repression could be observed in all cell lines, however, with different efficiency. In HEK293 TN cells, only intermediate reduction of ZsGreen expression was detected, whereas in HeLa and CHO cells repression down to background niveau was achieved. Strikingly, CHO cells display a distinct ZsGreen negative population of almost 40% and the smallest fraction of unaffected cells.

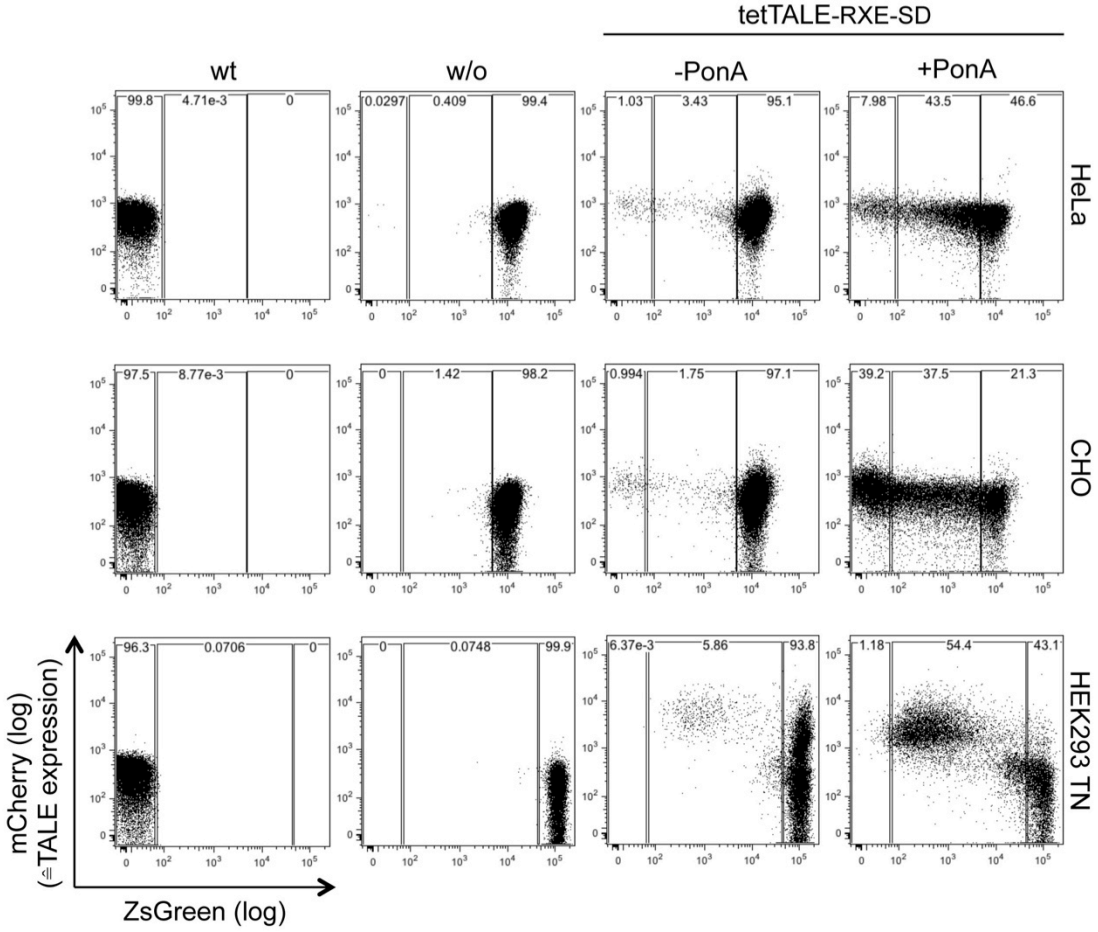

Figure 4.33 Stable repression of a chromosomal target by EF tetTALE-RXE-SD in three cell lines
HeLa, CHO and HEK293TN tetEF-ZsGreen clones were stably transfected with EF tetTALE-RXE-SD. Cells were then cultured in the presence or absence of 10 µM PonA for 7 days and ZsGreen reporter expression was monitored via FACS. Wildtype cells (wt) and untransfected tetEF-ZsGreen cells (w/o) served as controls. Shown is the analysis of stable cell pools.

It was decided to test the capacity of the regulatory system for stable repression with CHO cells. Therefore, an approach with single clones under constant PonA treatment was chosen, as FACS sorting previously proofed to be inefficient. To this end, a limited dilution of CHO tetEF-ZsGreen cells, stably transfected with tetTALE-RXE-SD and cultured with PonA for 13 days, was set up. Clones were selected for complete repression of the ZsGreen reporter and then subjected to 7 days of PonA removal, followed by 7 days of PonA addition. In contrast to the results obtained with HAFTL cells, FACS analysis demonstrated a stably repressed ZsGreen negative population of about 95% that was homogenously re-expressing the reporter upon the removal of PonA. Subsequent reinitiating of repression, by culture in the presence of PonA, resulted in a homogenous decrease of ZsGreen expression to background level.

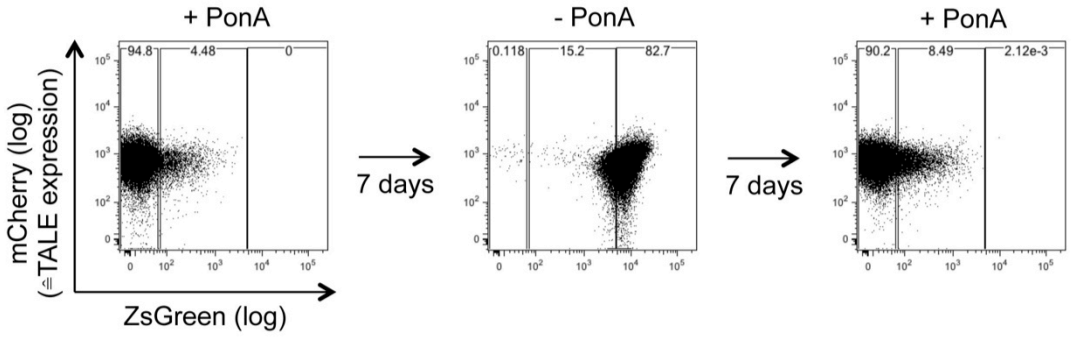

Figure 4.34 Reversibility of EF tetTALE-RXE-SD-mediated repression in CHO cells
CHO tetEF-ZsGreen EF tetTALE-RXE-SD were subjected to a limited dilution with constant presence of 10 µM PonA. Single clones were selected according to the absence of ZsGreen signal (left) and PonA was removed from the culture for seven days (middle), followed by seven days of culture in the presence of 10 µM PonA (right). ZsGreen reporter expression was analyzed by FACS. Shown is the analysis of a representative clone.

In summary, upon several experimental adjustments, the retinoid X/ ecdysone receptor gene switch has proven to be suitable to make tetTALE-SD-mediated repression inducible. Moreover, dose-dependent repression could be demonstrated. As no additional construct needs to be delivered, this single chain system represents a valuable tool to tune the repression of endogenous genes.

4.6 Competition of TALEs with other transcription factors for identical binding sites

As the previous experiments have demonstrated, tetTALE-SD is exceptionally potent in repressing the transcription from the strong human EF1α promoter. Next it was addressed how tetTALE performs when competing with other TFs for the same binding site. Two scenarios are conceivable: (a) tetTALE is bound to a target sequence and challenged by another TF. (b) The target site is occupied by another transcription factor and tetTALE has to displace it. Both settings can be analyzed by taking advantage of the inducible nature of the Tet system. X1/5 cells, carrying chromosomal copies of both P_{tet7} luc and tTA genes [30], were either cultured in the OFF state where tTA is not bound to *tet*O and the reporter is inactive (+dox), or in the ON state where tTA is bound to *tet*O and reporter expression is induced (-dox). The cells were stably transfected with either tetTALE-SD or tetTALE without repression domain under both starting conditions. To confirm and quantify their expression, either the T2A coupled EGFP expression was analyzed by FACS or the presence of the tetTALE itself was assessed by immunoblotting. The functionality of the T2A-mediated protein cleavage was checked by immunoblotting, using a EGFP-specific antibody. As depicted in Figure 4.35, tetTALE-SD and tetTALE were both homogenously expressed, independent on the dox conditions. EGFP detection by immunoblotting showed that the T2A site was functional and GFP was no longer coupled to tetTALE. This is evidenced by a single band at about 30 kDA (see Figure 4.35 B right blot).

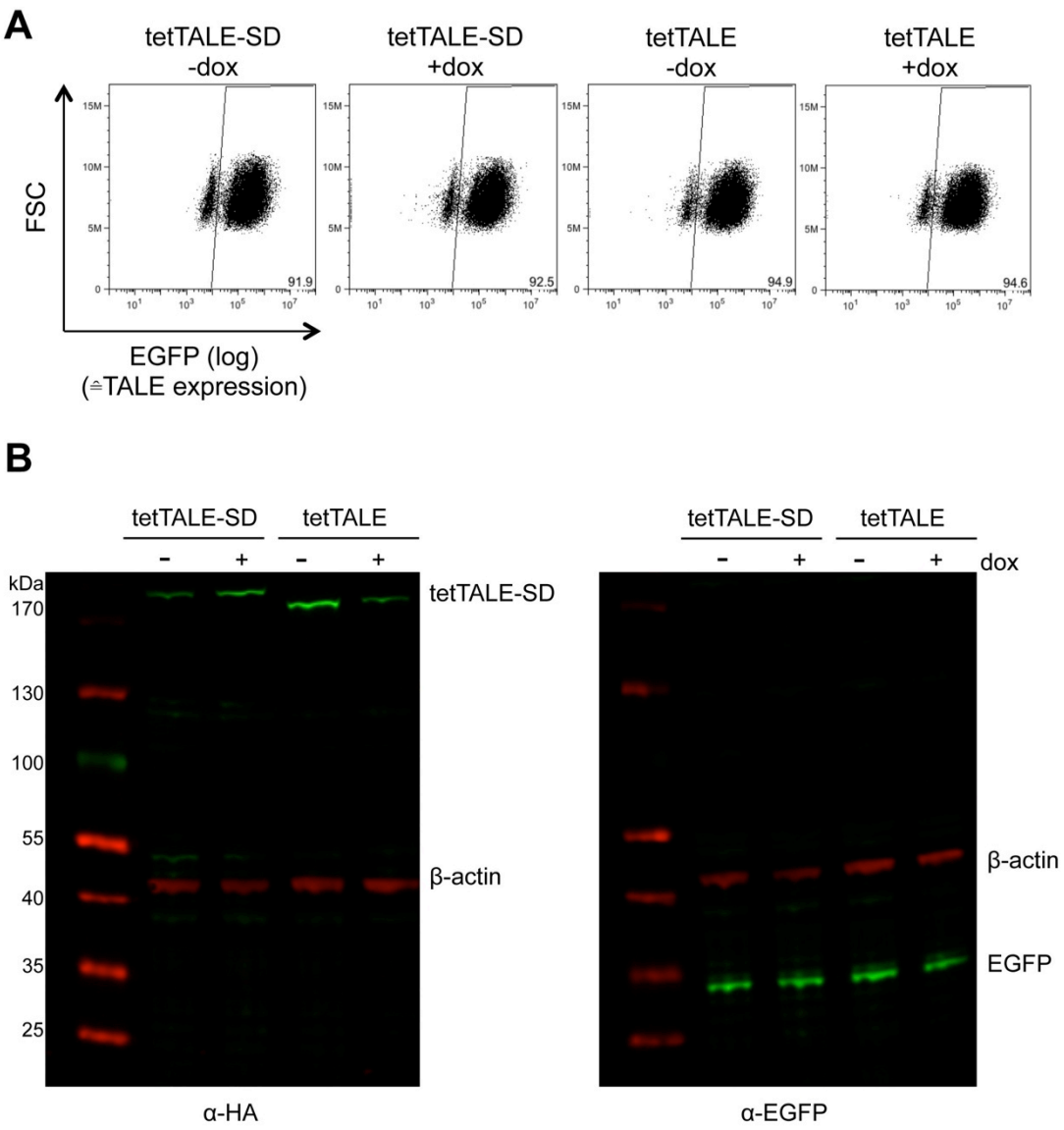

Figure 4.35 tetTALE(-SD) expression levels in X1/5 cells
(A) X1/5 cells were cultured in the absence (-dox) or in the presence (+dox) of doxycycline and stably transfected with either a tetTALE-SD or a tetTALE expression construct. T2A coupled EGFP expression in stable pools was analyzed by FACS. Shown is the analysis of stable cell pools.
(B) tetTALE(-SD) expression was monitored by immunoblotting with a α-HA antibody (left blot). Additionally, EGFP protein levels were determined by immunoblotting to check for T2A functionality with a α-EGFP antibody (right blot). β-actin levels served as a loading control. Shown is the analysis of stable cell pools.

The first scenario introduced, i.e. tetTALE is bound to a target sequence and challenged by another TF, was investigated. X1/5 cells were constantly cultured in the presence of dox in the OFF state, where tTA is not bound to the *tet*O and the reporter is inactive. Under these continued conditions the cells were stably transfected with either tetTALE-SD or tetTALE without repression domain, both capable of binding to *tet*O independent on the state of the Tet system. Subsequently, the Tet system was switched to the ON state by the removal of dox, theoretically enabling tTA to bind to the now occupied *tet*O (see Figure 4.36).

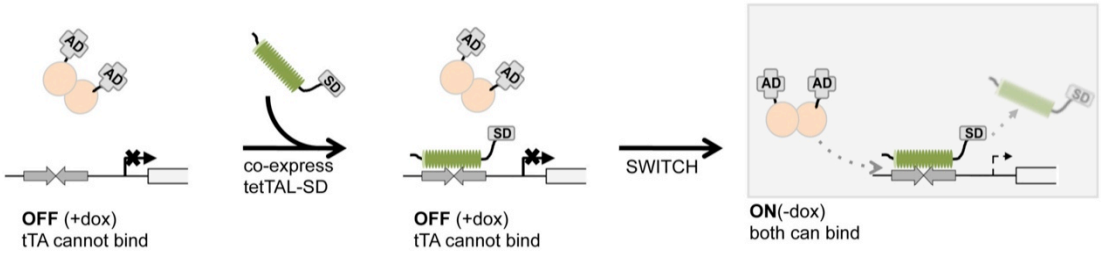

Figure 4.36 Experimental setup: Competition of pre-bound tetTALE/ tetTALE-SD with tTA for the same binding site
X1/5 cells, carrying chromosomal copies of the P_{tet7} luc reporter and a tTA expression cassette, were cultured under OFF conditions (+dox). Cells were stably transfected with either a tetTALE or a tetTALE-SD expression construct containing a T2A linked EGFP marker. In the OFF condition only the tetTALE(-SD) can occupy *tet*O. The Tet system was subsequently switched from OFF to ON, enabling tTA binding to *tet*O.
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Analysis of the luciferase reporter activity addressed if tTA was able to reestablish itself as the dominant transcription factor. Compared to untransfected X1/5 cells, tetTALE-SD transfected cells showed an almost 20-fold reduction in reporter activity in the ON state. To discriminate the effect of competitive binding from that of the silencing domain, tetTALE lacking an effector domain was used. The fact that reporter activity was reduced almost 10-fold in the presence of tetTALE, argues for a quantitative prevention of tTA binding rather than for the effect of a few *tet*O bound silencers (see Figure 4.37 A). To examine if protein size accounts for the difference between tetTALE-SD and tetTALE-mediated activation hindrance, the silencing domain (14kDa) was substituted for the more bulky mCherry fluorescence protein (27 kDa). As shown in Figure 4.37 B, the addition of the mCherry protein only slightly enhanced the inhibitory effect of tetTALE binding to *tet*O, which might be a contributor to the observed difference in activation hindrance with and without the silencing domain. Microscopic analysis of tetTALE-mCherry cells verified the nuclear localization of the TALE.

In summary, tetTALE is able to quantitatively prevent the binding of another transcription factor when pre-bound to an identical target site.

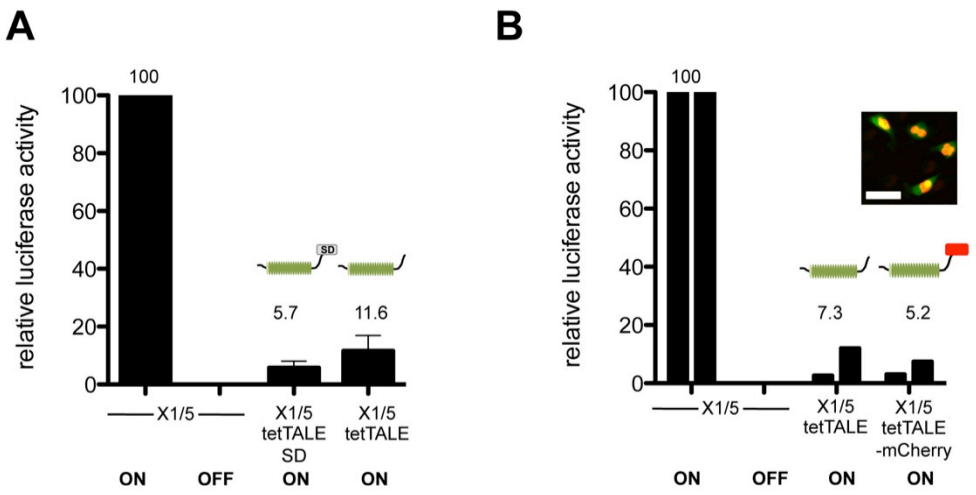

Figure 4.37 Competition of pre-bound tetTALE/ tetTALE-SD with tTA for the same binding site
(A) X1/5 cell pools, stably transfected with either tetTALE or tetTALE-SD in the OFF state, were harvested 7 days after the switch of doxycycline conditions and luciferase activity was analyzed. P_{tet7}-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only, was set to 100. Shown are mean values of three independent experiments with standard deviation.
(B) X1/5 cell pools, stably transfected with either tetTALE or tetTALE-mCherry, were harvested 7 days after the switch of doxycycline conditions and luciferase activity was analyzed. P_{tet7}-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only, was set to 100. Shown are results from two independent experiments for stably transfected X1/5 cell pools. Insert: microscopic picture of cells stably transfected with tetTALE-mCherry illustrating nuclear localization of TALE. Scale bar: 50 µm
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For testing the second scenario, if tetTALE can displace pre-bound tTA at its binding site, X1/5 cells were constantly cultured in the ON state (-dox) where tTA is bound to *tet*O and the reporter is active. Transfection with either tetTALE or tetTALE-SD addressed if both are capable of reversing tTA-mediated activation (see Figure 4.38).

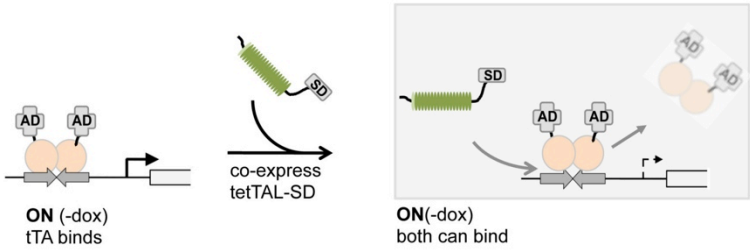

Figure 4.38 Experimental setup: Competition of pre-bound tTA with tetTALE/ tetTALE-SD for the same binding site.
X1/5 cells, carrying chromosomal copies of the P_{tet7} luciferase reporter and a tTA expression cassette, were cultured under ON conditions (-dox) where tTA is bound to *tet*O. Cells were stably transfected with either a tetTALE or tetTALE-SD expression construct containing a T2A linked EGFP marker.
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As demonstrated by luciferase activity measurements, tetTALE-SD is able to reduce tTA-mediated activation about 10-fold compared to untransfected X1/5 cells in the ON state. Again tetTALE lacking a silencing domain performs comparably, arguing for a displacement of tTA rather than a KRAB domain mediated silencing (see Figure 4.39 B). Once more the effect of protein size on this activation hindrance was tested by using tetTALE-mCherry. As depicted in Figure 4.39 B, an increasing protein size does not positively influence tetTALEs ability to counter tTA binding.

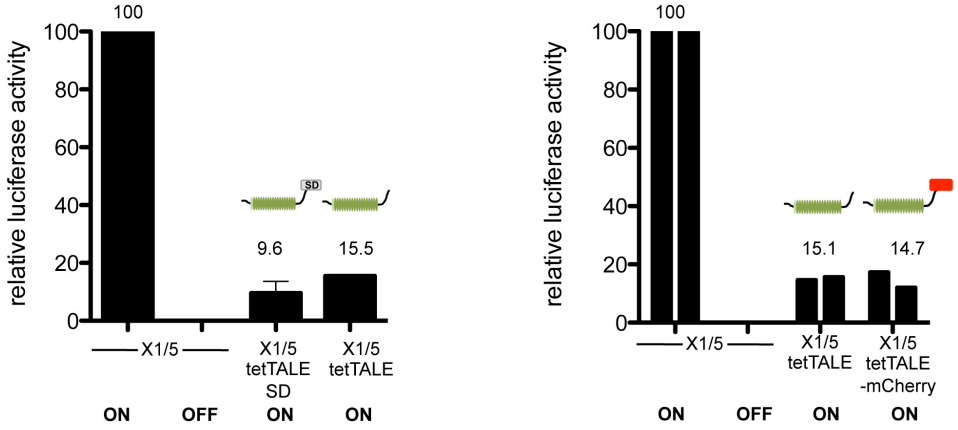

Figure 4.39 Competition of pre-bound tTA with tetTALE/ tetTALE-SD for the same binding site
(A) Luciferase activity was analyzed in X1/5 cell pools stably transfected with either tetTALE or tetTALE-SD in the ON state. P_{tet7}-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only, was set to 100. Shown are mean values of three independent experiments with standard deviation.
(B) Luciferase activity was analyzed in X1/5 cell pools stably transfected with either tetTALE or tetTALE-mCherry in the ON state. P_{tet7}-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only was set to 100. Shown are results from two independent experiments for stably transfected X1/5 cell pools.
Partly published in Werner & Gossen, 2014 [108]

This time, the analysis was extended by the testing of single clones for a correlation between tetTALE-SD /tetTALE expression and the decrease in luciferase reporter activity. Remarkably, tTA-mediated activation was particularly reduced in clones showing a high expression level of tetTALE-SD, either assessed by T2A coupled EGFP expression or immunoblot analysis. tetTALE-SD expressing clone 4 for example displayed the highest EGFP signal and a clear tetTALE-SD protein expression and a corresponding low luciferase activity in the ON state. In contrast clone 1 and 5, with only marginal EGFP signal and an undectable level of tetTALE-SD protein, showed luciferase activity highly similar to that of untransfected X1/5 cells. The same correlation was observed in tetTALE expressing X1/5 cells where clone 22 was one of the best performing clones regarding both tetTALE linked EGFP expression and reduced luciferase activity.

Taken together, these results demonstrate that the DNA-binding domain of tetTALE is able to prevent the binding of other transcription factors or actively compete with pre-bound TFs in a dose-dependent manner.

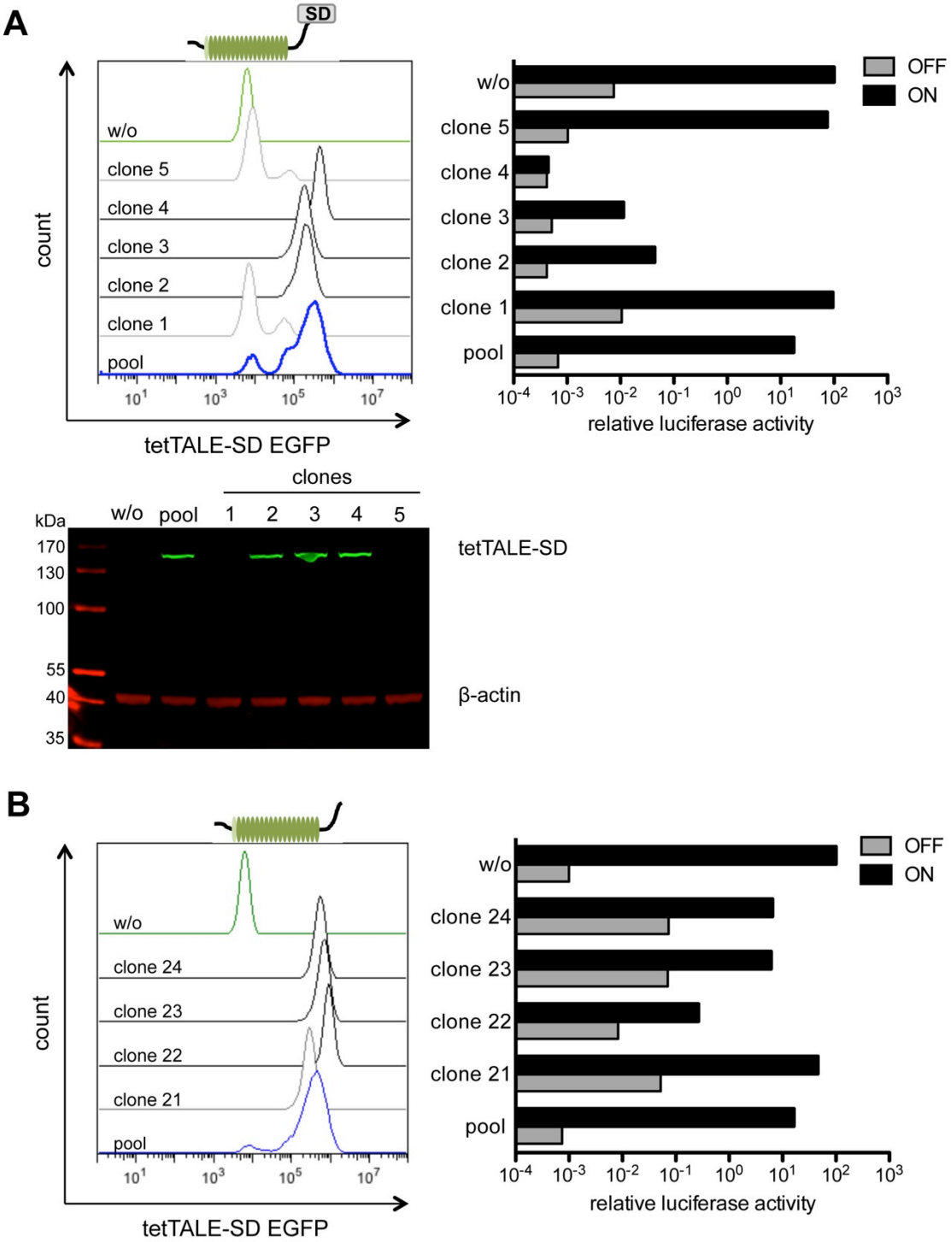

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Figure 4.40 Clonal analysis of the competition of pre-bound tTA with tetTALE/ tetTALE-SD for the same binding site
(A) Clones isolated from tetTALE-SD transfected X1/5 cells were analyzed for tetTALE-SD linked GFP expression along with untransfected X1/5 cells (w/o) and the originating pool (upper left panel). The same clones grown under ON conditions were analyzed for luciferase activity 7 days after the switch of doxycycline conditions from ON (-dox) to OFF (+dox). P_{tet7}-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only was set to 100 (upper right panel). Immunoblot analysis of single clones grown under ON conditions was performed to detect tetTALE-SD expression levels. A α-HA antibody was used and β-actin levels served as loading control (lower panel).
(B) Clones isolated from tetTALE transfected X1/5 cells were analyzed for tetTALE linked GFP expression along with untransfected X1/5 cells (w/o) and the originating pool (left panel). The same clones grown under ON conditions were analyzed for luciferase activity 7 days after the switch of dox conditions from ON (-dox) to OFF (+dox). P_{tet7}-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only was set to 100 (right panel). Partly published in Werner & Gossen, 2014 [108]

To substantiate that the efficacy of tetTALE in these binding site competition assays is not a mere cell type or reporter-specific artifact, an extended and slightly modified experiment was performed in engineered CHO and HEK 293 cells. CHO K1 Tet-On Advanced and HEK 293 Tet-On Advanced cells carry an expression cassette for the reverse tet *trans*-activator (rtTA). Furthermore, they were stably transfected with a construct containing a bidirectional tet-responsive promoter driving luciferase and EGFP as reporter genes ($P_{tet7(bi)}$ EGFP/luc; J.Contzen and M. Gossen, unpublished). EGFP enables a single cell resolution of the competition. Like in the previous experiment, the cells were cultured in the OFF state and stably transfected with either tetTALE-SD or tetTALE. Subsequently, the dox conditions were switched and the expression of both reporter genes was quantified (see Figure 4.41).

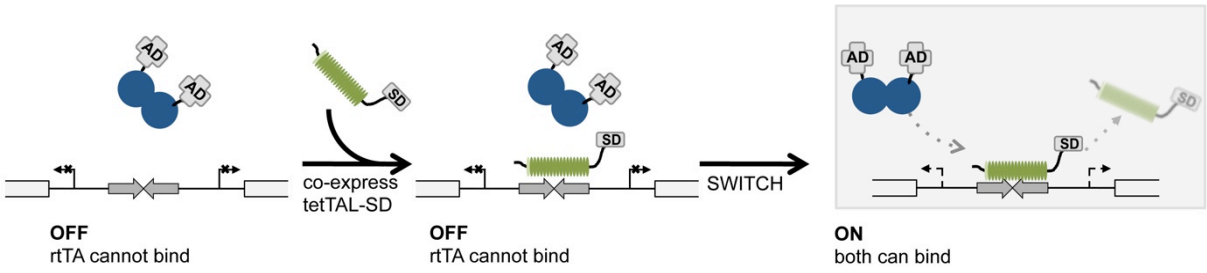

Figure 4.41 Experimental setup: Competition of pre-bound tetTALE/ tetTALE-SD with rtTA for the same binding site
CHO K1 Tet-On Advanced and HEK 293 Tet-On Advanced cells, containing chromosomal copies of a bidirection P_{tet7} luciferase and EGFP reporter and a rtTA expression cassette, were cultured under OFF conditions. Cells were stably transfected with either a tetTALE or tetTALE-SD expression construct containing a T2A linked mCherry marker. In the OFF condition only the tetTALE(-SD) can occupy *tet*O. The Tet system was subsequently switched from OFF to ON, enabling rtTA binding to *tet*O.
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As depicted in Figure 4.42, the results from both luciferase and EGFP measurments confirmed earlier findings. For both cell lines a substantial reduction in luciferase activity was observed in tetTALE transfected cells compared to parental cells in the ON state. Again, the silencing domain proofed to have only a marginal effect, arguing for an effective hindrance of rtTA binding. FACS analysis showed a clear correlation between tetTALE linked mCherry expression and the reduction of EGFP signal. For tetTALE transfected CHO cells more than 80 % showed a reduction of EGFP expression to background level and a bright mCherry signal, indicative of high tetTALE protein levels. The complete abrogation of EGFP signal proofed that tetTALE can totally prevent rtTA binding. For HEK 293 cells still 50% of the cells exhibited a marked reduction of EGFP signal, although to a lesser extent than observed in CHO cells. This is in line with the luciferase results where CHO also outperformed HEK 293 cells.

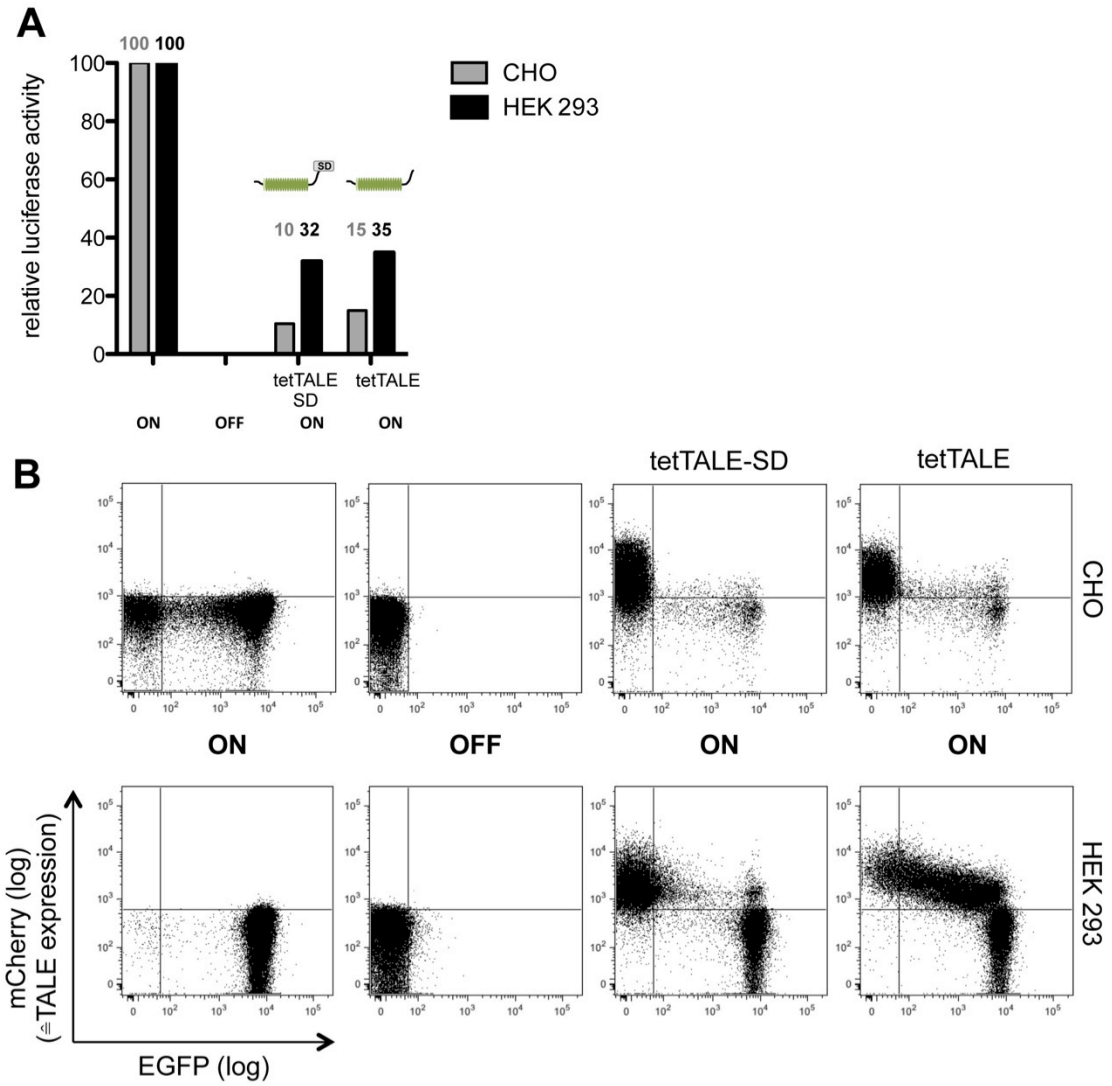

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Figure 4.42 Competition of pre-bound tetTALE/ tetTALE-SD with rtTA for the same binding site
(A) CHO K1 Tet-On Advanced and HEK 293 Tet-On Advanced pools, stably transfected with $P_{tet7(bi)}$ EGFP/luc and either tetTALE or tetTALE-SD in the OFF state, were harvested 7 days after the switch of dox conditions and luciferase activity was analyzed. $P_{tet7(bi)}$-mediated luciferase activation of the TALE negative parental cell line with bound tet activator only was set to 100.
(B) Cell pools stably transfected with either tetTALE or tetTALE-SD were harvested 7 days after the switch of dox conditions. Expression of EGFP and mCherry (coupled to tetTALE(-SD) via T2A) was analyzed by FACS.
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The second setting where tetTALE is faced with an occupied target sequence was tested in an analogous approach. CHO K1 Tet-On Advanced and HEK 293 Tet-On Advanced cells were cultured in the ON state where rtTA is bound and both reporter genes are expressed. After tetTALE or tetTALE-SD were stably transfected, reporter activity was analyzed (see Figure 4.43).

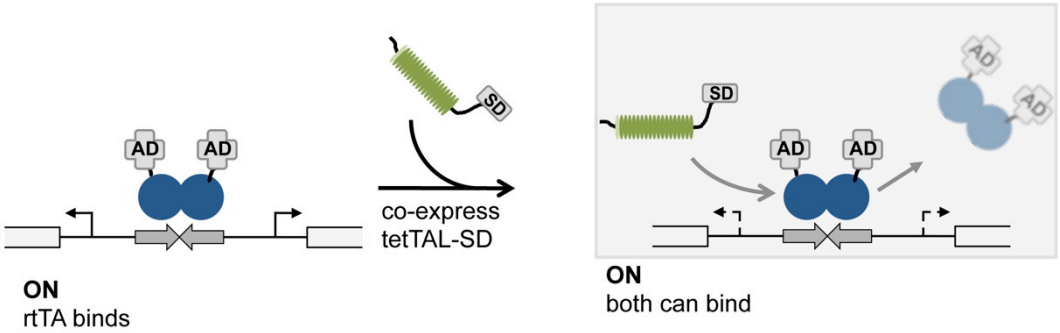

Figure 4.43 Experimental setup: Competition of pre-bound rtTA with tetTALE/ tetTALE-SD for the same binding site
CHO K1 Tet-On and HEK293 Tet-On cells, containing a bidirectional P_{tet7} luciferase and EGFP reporter, and a rtTA expression cassette stably integrated, were cultured under ON conditions where rtTA is bound to *tet*O. Cells were stably transfected with either a tetTALE or a tetTALE-SD expression construct containing a T2A linked mCherry marker.
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In accordance with findings in X1/5 cells, tetTALE can effectively displace pre-bound rtTA, resulting in a distinct reduction of reporter expression. This holds true for both cell lines tested. However, the effect was again more pronounced in CHO cells (see Figure 4.44 A, B).
Microscopic analysis in HEK 293 cells clearly showed that EGFP signal was reduced with increasing mCherry expression, confirming FACS analysis data (see Figure 4.44 C).

As a summary, tetTALE was able to compete with tTA as well as rtTA for an identical binding site independent on the cell or reporter system. There is a good case to believe that this competitive potential is more universal and applicable beyond the Tet system.

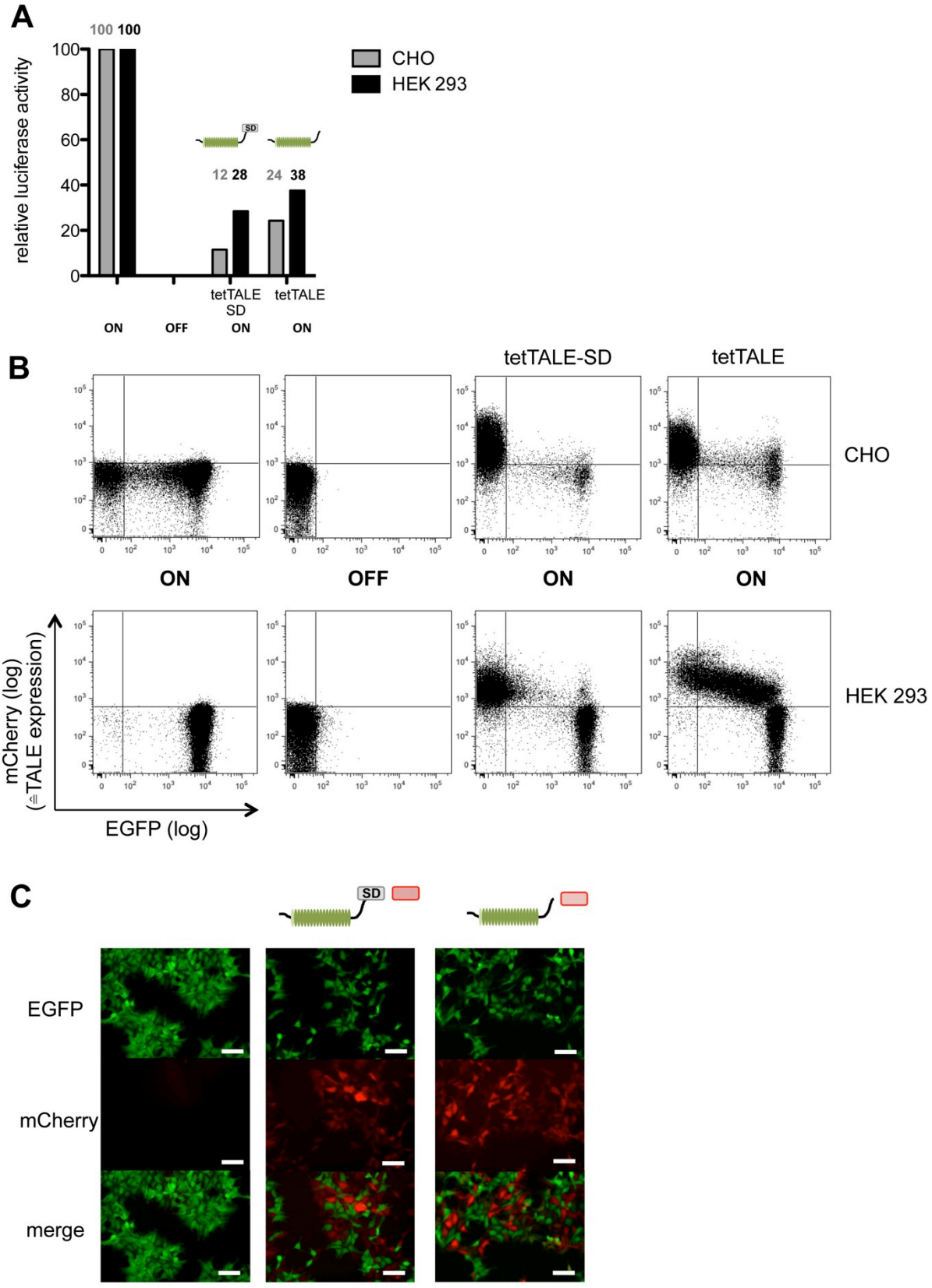

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Figure 4.44 Competition of pre-bound rtTA with tetTALE/ tetTALE-SD for the same binding site
(A) Luciferase activity was analyzed in CHO K1 Tet-On Advanced and HEK 293 Tet-On Advanced cell pools, stably transfected with $P_{tet7(bi)}$ EGFP/luc and either tetTALE or tetTALE-SD in the ON state. $P_{tet7(bi)}$-mediated luciferase activation of the TALE negative parental cell lines with bound tet activator only was set to 100.
(B) Expression of EGFP and mCherry (coupled to TALE via T2A) was analyzed by FACS in cell pools stably transfected with either tetTALE or tetTALE-SD.
(C) Microscopic picture of HEK293 Tet-On cells, containing a bidirectional P_{tet7} luciferase and a EGFP reporter, and a rtTA expression cassette, before and after stable transfection with tetTALE/ tetTALE-SD: EGFP (top), mCherry (middle), merge (bottom). Scale bar: 50 µm.
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4.7 Interference with transcription initiation by TALEs

As a next step, the competitive abilities of tetTALE were further characterized. It was demonstrated that TALEs can efficiently maintain target binding in the presence of other TFs sharing the same specificity and competitively displace them. Subsequent experiments focused on competition with the basal transcription machinery at the site of transcription initiation. Therefore, the commercially available reporter construct CMV $tetO_2$ EGFP was used where the CMV promoter is equipped with two *tet*O sequences proximal to the TATA box, enabling tetR-mediated downregulation (T-Rex system) [123]. The question was addressed if tetTALE binding to the two *tet*O sites, partially overlapping with the transcription initiation region, resulted in a repression of EGFP reporter expression (see 4.45 A). The first double transient experiments were performed with different ratios of reporter: repressor (w/w) to take stoichiometric aspects into account. As depicted in Figure 4.45 B and C, co-transfection of reporter and tetTALE resulted in a distinct reduction of EGFP expression up to 10-fold, an extent comparable to that achieved by tetR. This downregulation was observed for all tested ratios, suggesting a high competitive potential with complex protein assemblies.

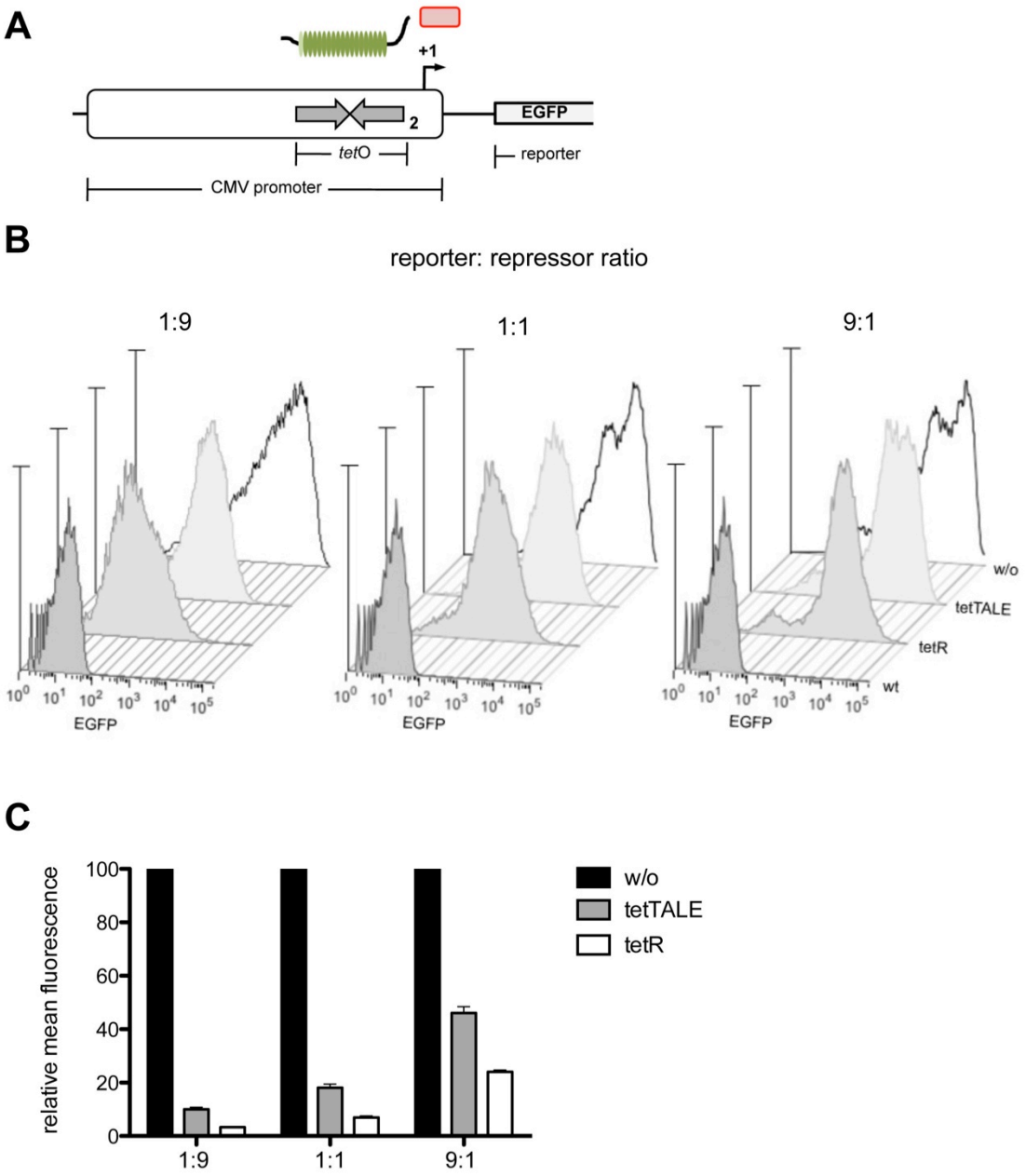

Figure 4.45 tetTALE-mediated inhibition of transcription initiation
(A) Scheme of the CMV tetO_2 EGFP reporter construct where two copies of *tet*O are located between the TATA box and the transcriptional start site of a CMV promoter.
(B) Overlay of histograms of 293HEK TN cells transiently transfected with the reporter alone (w/o), or co-transfected with either tetTALE or tetR in different w/w ratios. Untransfected cells served as a control (wt). Shown is the FACS analysis of a representative transfection.
(C) Quantification of the fluorescence signal of CMV reporter activity in transfected EGFP positive cells. The signal intensity of cells transfected with the reporter alone was set to 100. Shown are mean values of three independent transfections with standard deviation.
Partly published in Werner & Gossen, 2014 [108]

Potential strand-specific effects in this competition assay were tested by comparing tetTALE and tetTALE$_{rev}$, binding to the opposite strand of *tet*O. Analysis of EGFP expression via FACS showed that tetTALE orientation had only a minor effect, with

targeting the sense strand resulting in a slightly better suppression of reporter transcription.

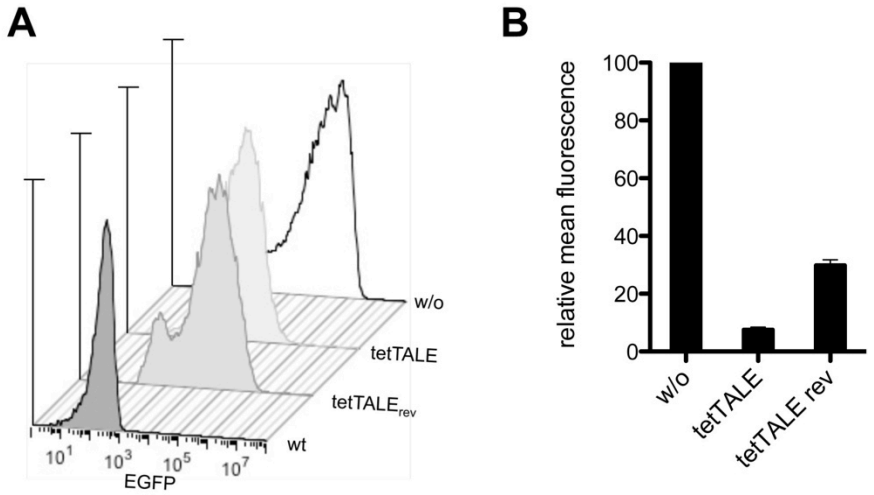

Figure 4.46 Strand dependency of tetTALE-mediated inhibition of transcription initiation
(A) Overlay of histograms of 293HEK TN cells transiently transfected with the reporter alone (w/o), or co-transfected with either tetTALE or tetTALE$_{rev}$ (1:9 w/w). Untransfected cells served as a control (wt). Shown is the FACS analysis of a representative transfection.
(B) Quantification of the fluorescence signal of CMV reporter activity in transfected EGFP positive cells. Signal intensity of the cells transfected with the reporter alone was set to 100. Shown are mean values of three independent experiments with standard deviation.

To extend the findings to another cell line and to assess the durability of the downregulation, CHO K1 and HEK293 TN cells were co-transfected with the reporter and tetTALE. EGFP reporter expression was determined on day 2, 3 and 4 after transfection. The previously detected repression in HEK293 TN cells was confirmed in CHO cells, despite lower transfection efficiency. The downregulation was stable over several days in both cell lines. Microscopic analysis reflected the results from FACS analysis, namely that high tetTALE linked mCherry levels correlated with low EGFP reporter expression (see Figure 4.47).

Taken together, these results demonstrate that at least in double transient experiments tetTALE is capable of efficiently interfering with the initiation of transcription. This is achieved by steric hindrance without the assistance of transcriptional silencing domains when the target site overlaps with the pre-initiation complex binding region.

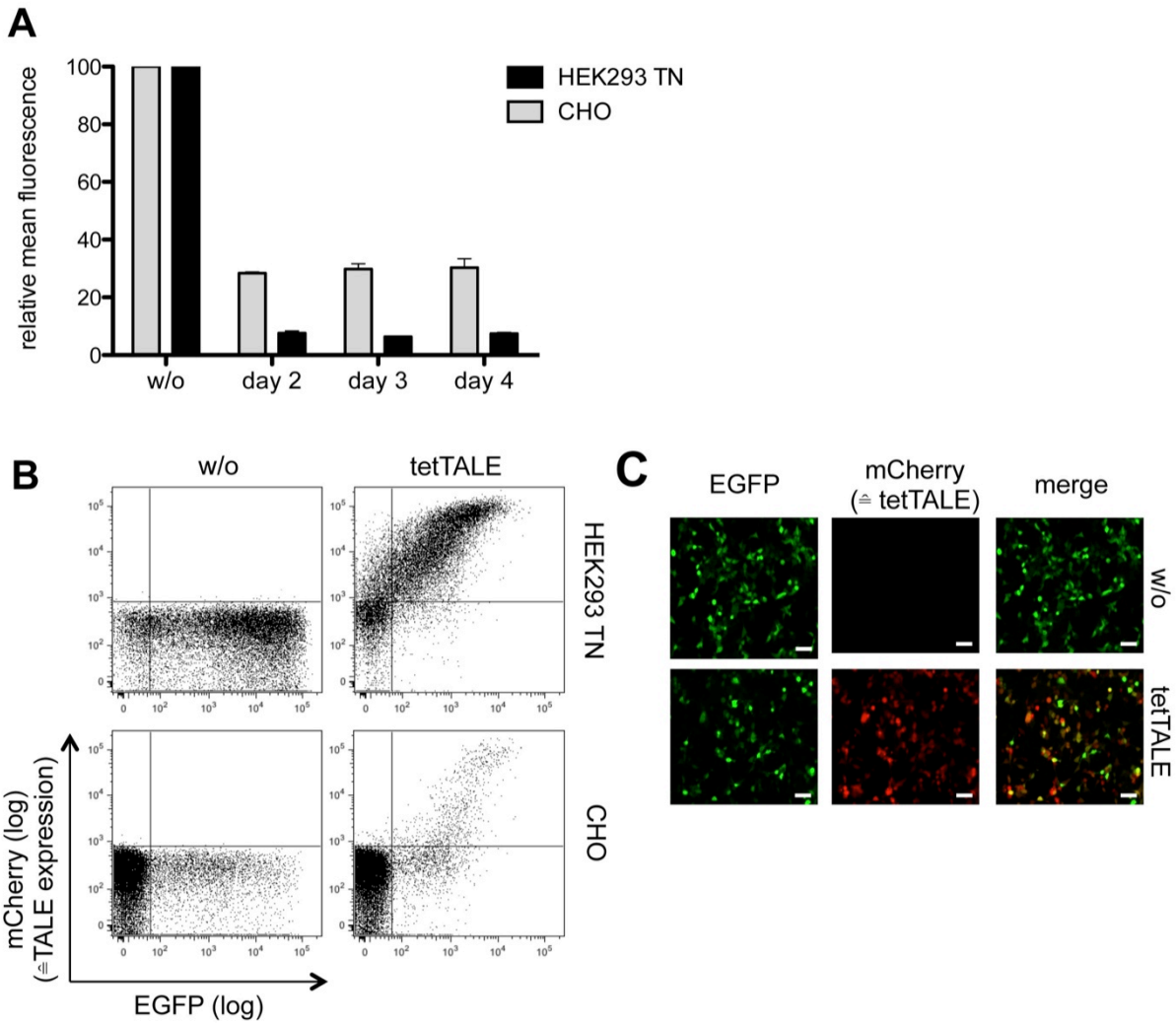

Figure 4.47 Time course of tetTALE interference with transcription initiation
(A) Quantification of the fluorescence signal of CMV reporter activity in HEK293 TN and CHO cells co-transfected with the reporter and tetTALE. Transfected EGFP positive cells were analyzed at different time points. Signal intensity of the cells transfected with the reporter alone was set to 100. Shown are mean values of three independent experiments with standard deviation
(B) Analysis of EGFP and mCherry (coupled to tetTALE via T2A) expression in HEK293 TN and CHO K1 cells transiently transfected with the reporter alone (w/o) or co-transfected with tetTALE (1:9 w/w) two days after transfection. Shown is the FACS analysis of a representative transfection.
(C) Microscopic picture of HEK293 TN cells after transient transfection with the reporter alone (w/o) or co-transfected tetTALE two days after transfection: EGFP (left), mCherry (middle), merge (right) Scale bar: 50 µm. Shown is the microscopic analysis of a representative transfection.

Double transient repression experiments face the problem that reporter expression coincides with the expression of the repressor. Thus, the repression capacity can easily be underestimated. Therefore, a double stable experiment was set up where reporter and tetTALE were both stably expressed in HEK293 TN cells. The CMV tetO2 EGFP reporter was integrated first and a single clone was selected with regard to high and uniform reporter expression. Subsequently, this clone was stably transfected with a tetTALE expression cassette. Contrary to expectations, the tetTALE-mediated inhibition of transcription turned out to be lower than what was

observed in double transient experiments. All cells showed a clear tetTALE linked mCherry signal and a homogenous decrease of EGFP expression, but only by a factor of 5.6. This was observed with the reporter cell pool as well as with two reporter clones of which one is shown in Figure 4.48.

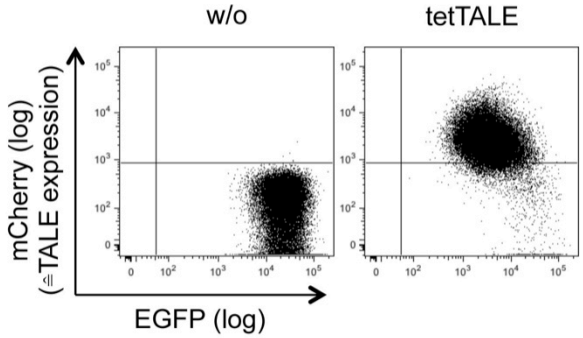

Figure 4.48 Stable tetTALE-mediated inhibition of transcription initiation
HEK293 TN cells were stably transfected with the CMV tetO_2 EGFP reporter and single clones were isolated. Subsequently, tetTALE coupled to mCherry was stably integrated in these clones. EGFP expression was assessed by FACS analysis in cells carrying the reporter only (w/o) and tetTALE transfected cells. Shown is the analysis of a representative clone.

Summing up, tetTALE is capable of interfering with the complex transcription initiation machinery and downregulating expression from the strong CMV promoter, although there is still room for improvement. Even when considering these quantitative limitations, this makes TALEs a versatile tool for the regulation of transcription when omission of silencing domains is desired.

4.8 Comparison of tetTALEs with *tet*O targeting dCas9-based transcription factors

During the course of this thesis, another system for tailor-made DNA-targeting emerged as an alternative to TAL effectors. In 2013, two groups adapted the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) type II system from *Streptococcus pyogenes* for applications as a genome editing tool in mammalian cells [79,80]. The system is based on a so-called single guide RNA (sgRNA), a fusion of crRNA and tracrRNA, which directs the endonuclease Cas9 to the target sequence, resulting in a double strand break. Several reports on Cas9 proteins with point mutations, disrupting nuclease activity, (dCas9) fused to transcriptional regulatory domains, illustrate the system's broad applicability for gene regulation [124-126]. To test this system in comparison to the TALE system, thoroughly analyzed in this thesis, a catalytically inactive dCas9 was

fused to a VP64 activation domain and linked to a mCherry reporter via a T2A site (dCas9-AD). Upon co-transfection with a suitable sgRNA, recruiting dCas-AD to a defined target sequence, the protein/RNA/DNA assembly is expected to stimulate transcription initiation, if appropriately localized in the context of a promoter (see Figure 4.49 A). sgRNAs targeting the *tet*O sequence have been previously reported in mammalian and yeast cells [124,127]. However, the sgRNA target sequence used by Cheng *et al.* [124] needed to be adjusted to the *tet*O_7 unit used in this study (tetOa). This was only possible by omitting the 5' G at the start of the sgRNA, otherwise providing the optimal start nucleotide for the U6 promoter. Additionally, a second sgRNA targeting a sequence a little further upstream was created (tetOb). The two sgRNA target sites used for all further experiments are depicted in Figure 4.49 B.

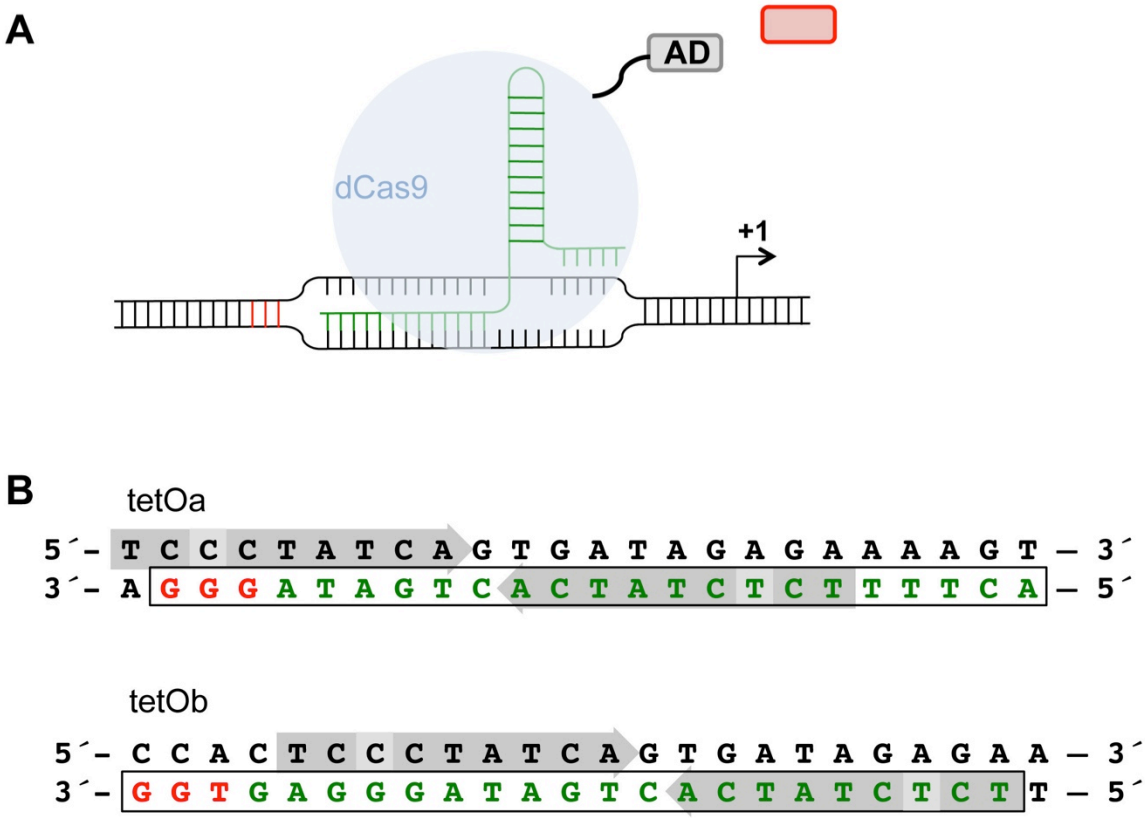

Figure 4.49 CRISPR/Cas-based activation
(A) Schematic representation of transcriptional activation with dCas9-AD. dCas9, harboring two point mutations disrupting its nuclease activity, is fused to an activation domain (AD) and a T2A linked mCherry reporter. In the presence of a sgRNA, recognizing a 20 bp sequence adjacent to a specific PAM sequence, the dCas9-AD complex is recruited to the target site and induces expression.
(B) Schematic representation of the sgRNA target sites in the *tet*O sequence. The target sequence is marked in green and the PAM sequence in red. Light grey boxes mark positions deviating from the operator symmetry.

For a first comparative, analysis a double transient activation assay was chosen. The two components of the CRISPR/Cas system were delivered on a single plasmid (see Figure 4.50 A). The previously used P_{tet7} luc reporter (see Figure 4.1) was co-transfected with tTA, tetTALE-AD or dCas9-AD with either gRNA in HeLa cells. Cells transfected with the reporter only or with dCas9-AD, but without corresponding sgRNA, served as a control. As shown in Figure 4.50 B, reporter activation achieved by both sgRNAs was in the same order of magnitude as what was detected for tetTALE-AD, with a maximum of 560-fold activation by tetOa sgRNA. Consequently, it is assumed that sgRNA expression is sufficient despite the change in the transcription initiation site. Moreover, a clear dependence on the presence of the sgRNA was shown, as transfection with dCas9-AD alone did not result in reporter expression.

As a next step, a similar experiment was performed in X1/6 cells, carrying copies of P_{tet7} luc stably integrated, to assess the efficiency on a chromosomal target. The measured activation rates followed exactly the same pattern as in the double transient experiment, albeit with overall lower fold activation.

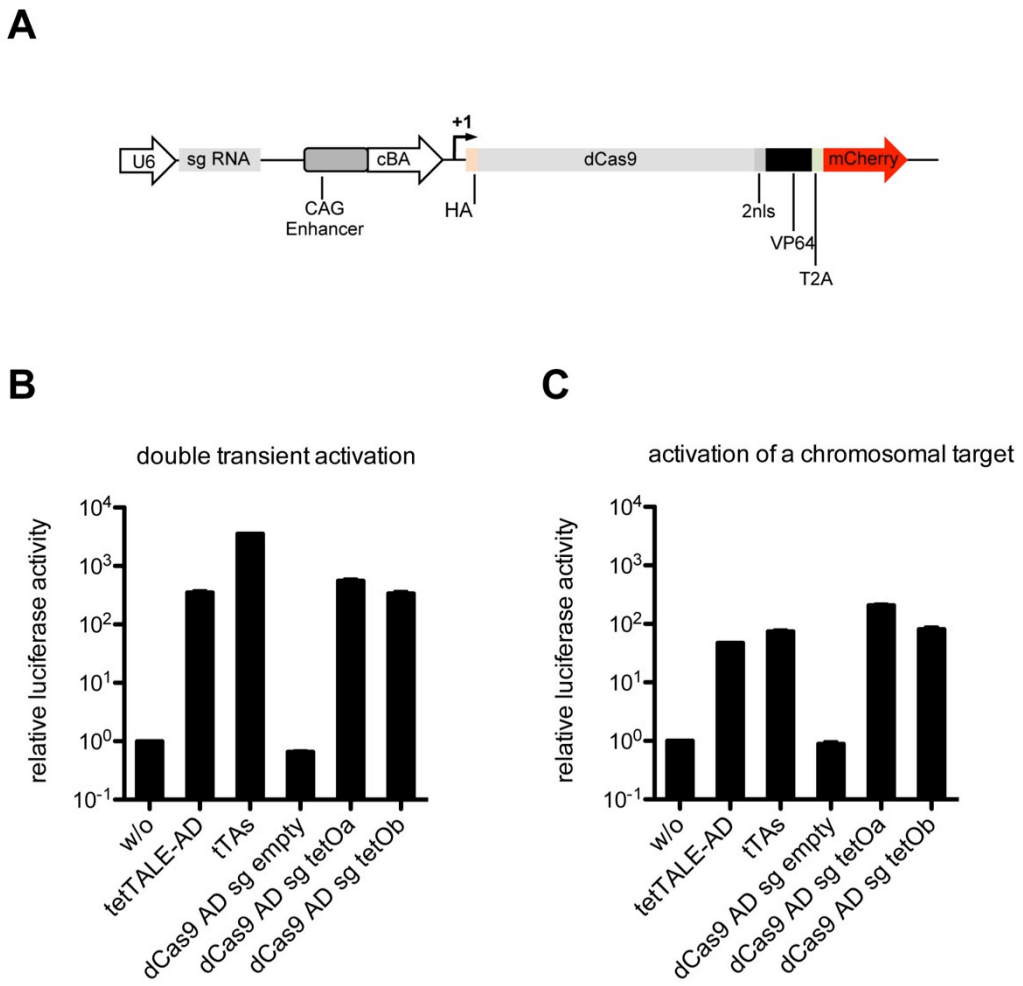

Figure 4.50 dCas-AD/sgRNA-mediated activation
(A) Schematic representation of the dCas9/ sgRNA expression vector..The catalytically inactive dCas9 is fused to a NLS sequence and a VP64 activation domain. Furthermore, a mCherry reporter is linked via a T2A site. The whole cassette is driven by a chicken β-actin promoter (cBA) with an upstream enhancer (CAG). The sgRNA expression cassette is located upstream and driven by a U6 promoter.
(B) HeLa cells were transfected with the P_{tet7} luc reporter and one activator expression construct. As a negative control for dCas9-AD-mediated expression it was transfected without sgRNA. A *Renilla* luciferase expression construct was included for internal standardization. P_{tet7} activity without activator was set to 1. Shown are mean values of three independent transfections with standard deviation.

Having shown that dCas9-AD in combination with a suitable sgRNA can induce strong reporter activation, RNA-specified effector domain recruitment was also tested in transcriptional repression. Hence, a dCas-SD cassette was constructed where the activation domain was replaced for a KRAB silencing domain. The sgRNA unit of the plasmid was left untouched. dCas9-SD with either of the two sgRNAs was co-transfected with pUHC13-13, harboring a constitutively active *tet*O modified CMV promoter driving a luciferase reporter (see Figure 4.9). Again, tetTALE-SD and tetR-SD were tested for comparison. For both cell lines analyzed, expression of dCas-SD alone without sgRNA did not result in a reduction of reporter activity. HEK293 TN

cells coexpressing dCas-SD and *tet*O sgRNAs lagged behind the performance of tetTALE-SD$^+$ cells by a factor of 4, resulting in an overall 10-fold reduction of reporter activity. Results from HeLa cells were even less convincing where tetTALE-SD performed 30-fold more efficient.

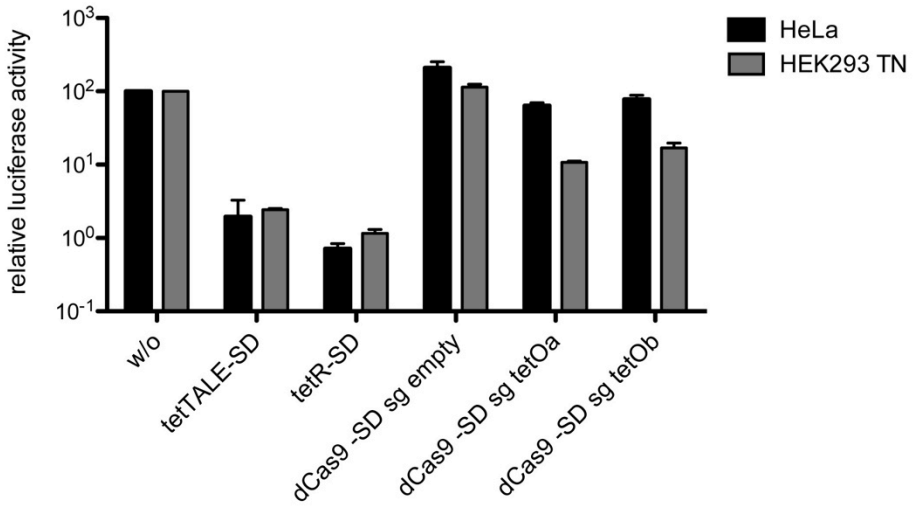

Figure 4.51 dCas-AD/sgRNA-mediated repression
HeLa and HEK293 TN cells were co-transfected with pUHC13-13 and one silencer expression construct. As a negative control for dCas9-SD-mediated expression, it was transfected without sgRNA. A *Renilla* luciferase expression construct was included for internal standardization. Reporter activity without repressor was set to 100. Shown are mean values of three independent transfections with standard deviation.

In a final transient transfection experiment, the capacity of dCas9/sgRNA without silencing domain to interfere with transcription initiation was tested in the same way as previously demonstrated for tetTALE (see Figure 4.45). The CMV tetO2 EGFP reporter construct was co-transfected with either dCas9 (without silencing domain) alone or with dCas9 in combination with one of the two sgRNAs. Despite the high dCas9 linked mCherry expression observed in all three transfections, neither sgRNA caused a decrease in EGFP reporter expression. In contrast to tetTALE, the complex of dCas9 and sgRNA was not efficient in inhibiting transcription initiation in the chosen experimental setting.

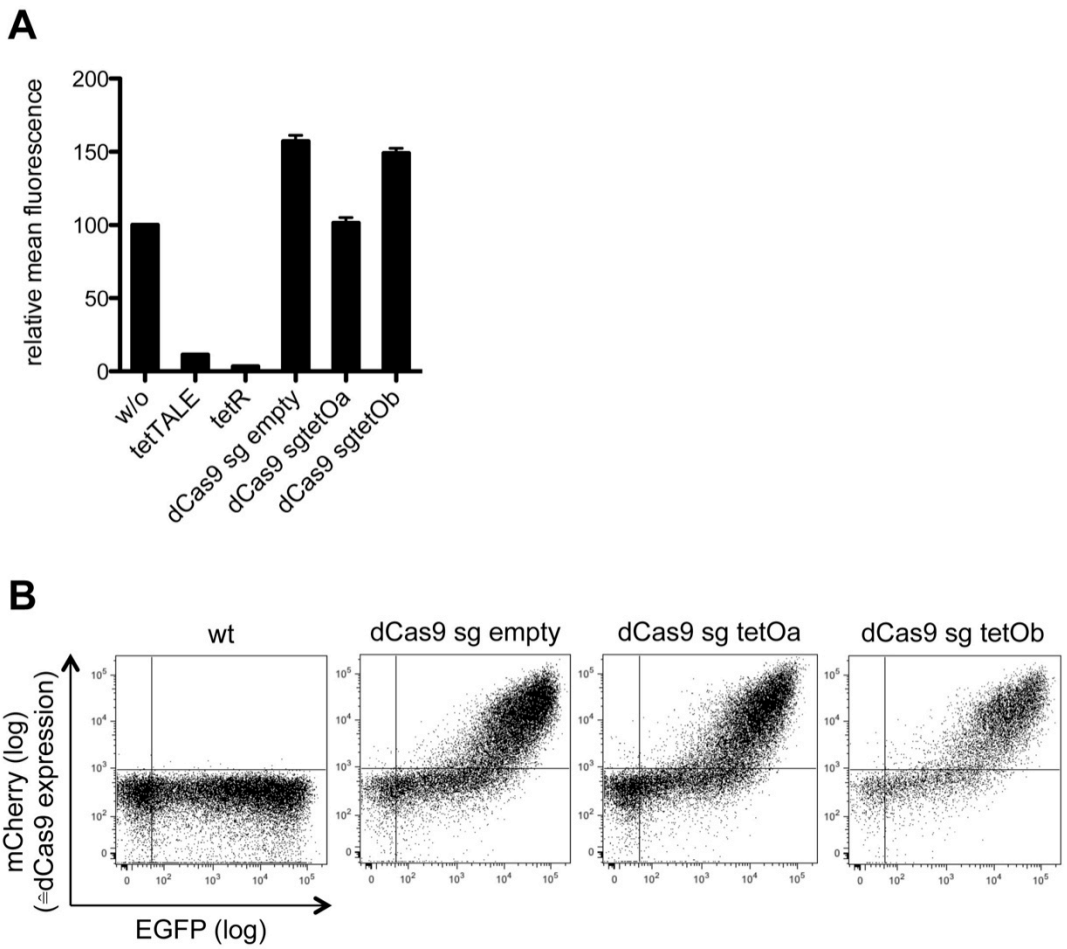

Figure 4.52 dCas9s/gRNA-mediated inhibition of transcription initiation
(A) Quantification of the fluorescence signal of CMV reporter activity in CMV tetO_2 EGFP transfected HEK293 TN cells. Signal intensity of the cells transfected with the reporter alone was set to 100. Shown are mean values of three independent transfections with standard deviation.
(B) Analysis of EGFP and mCherry (coupled to dCas9 via T2A) expression in HEK 293 TN cells transiently transfected with the reporter alone (w/o) or co-transfected with dCas9/sgRNA (1:9 w/w) two days after transfection. Shown is the FACS analysis of a representative transfection.

On the grounds of the encouraging sgRNA directed activation results, it was decided to test dCas9/sgRNA activation capacity in double stable experiments. HeLa cells carrying a chromosomal cassette of a tet-responsive promoter upstream of a ZsGreen reporter were used (see Figure 4.53 A). These cells were then transfected with either dCas9-AD alone as a negative control or dCas9-AD in combination with one of the two sgRNAs. Two days after transfection a fraction of the cells was analyzed by FACS for reporter activation and the rest was subjected to antibiotic selection. FACS analysis was conducted again after selection was completed. As shown in Figure 4.53 B, dCas9-AD in combination with the sg tetOa eventuated in a distinct population positive for dCas9-AD linked mCherry as well as for the activated ZsGreen reporter. Surprisingly, this was not observed with sg tetOb. Moreover, the transient activation did not reflect the stable situation where neither a mCherry nor a

ZsGreen signal was observed. These contradictory results led to the idea that expression levels of one of the components was to low upon stable integration. So HeLa cells, stably carrying the reporter and the dCas9-AD/sg tetOa cassette, were transiently supertransfected with either dCas9-AD alone, sg tetOa alone or both. Only a supertransfection with both components reconstituted the activation observed after initial transient transfection, arguing for an overall too low expression level of both components but the principal functionality of the approach.

In summary, the dCas9-AD/sg tetO activators acted comparable to tetTALE-AD and tTAs in transient activation experiments. Stable activation experiments require further optimization but were shown to be functional in general. Results from *trans*-repression experiments demonstrated that, under the selected conditions, tetTALE-SD clearly outperformed dCas9-SD. Lastly, interference with a large protein complex like the transcription initiation machinery could not be achieved with dCas9.

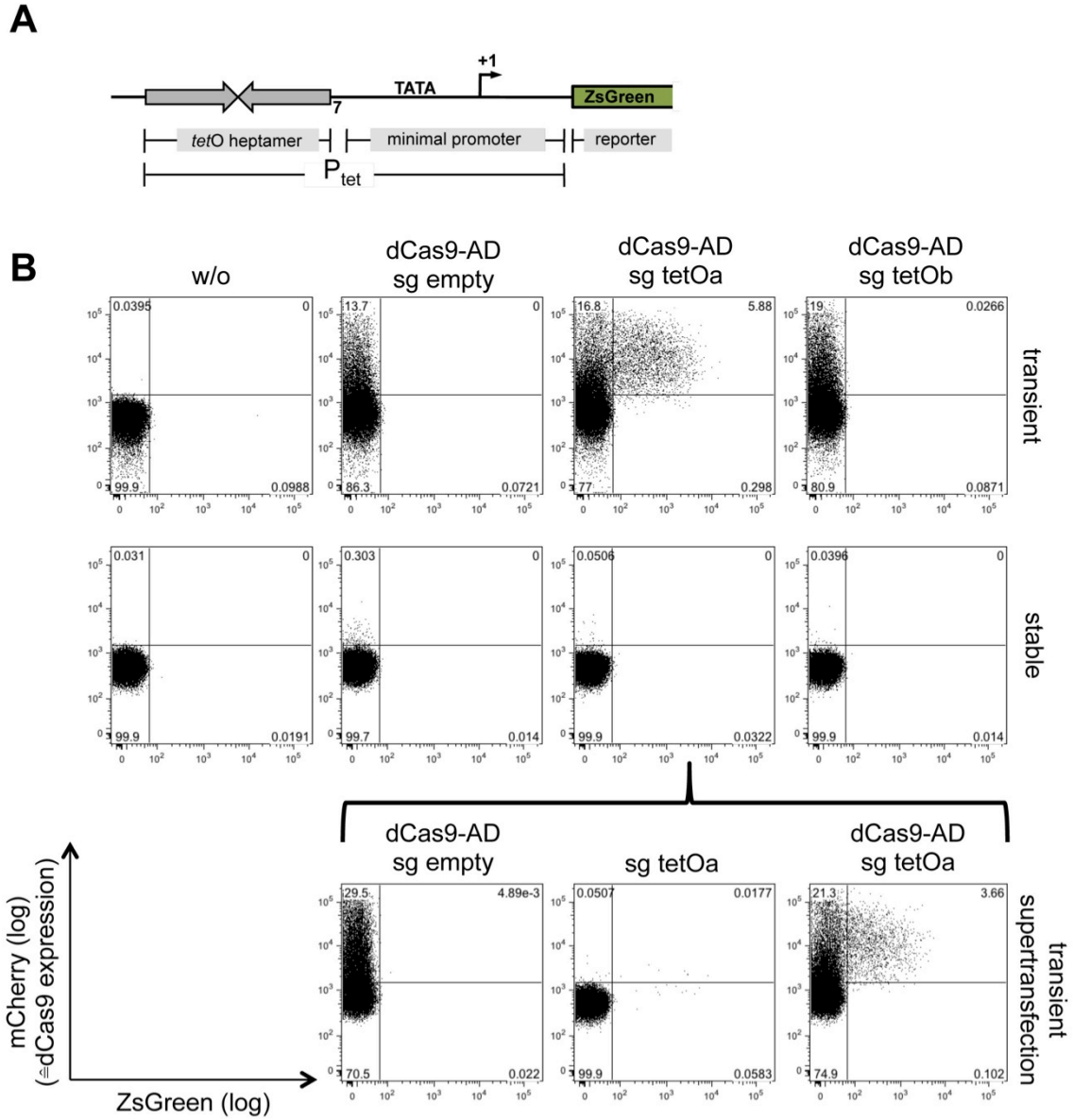

Figure 4.53 Stable dCas-AD/sgRNA-mediated activation
(A) Schematic representation of the reporter which was stably integrated in HeLa cells. A tet-responsive promoter is located upstream of a ZsGreen reporter (P_{tet7} ZsGreen) .
(B) HeLa P_{tet7} ZsGreen cells were transfected with dCas9-AD alone or in combination with either of the tetO sgRNAs. FACS analysis was performed two days after transfection and after the completion of selection for stable integration. Double stable HeLa P_{tet7} ZsGreen dCas9-AD/sg tetOa cells were transiently supertransfected with either dCas-AD, sg tetOa or both. FACS analysis was done two days after transfection. Shown is the analysis of stable cell pools

5 Discussion

The modulation of gene activity via regulatable transcriptional control has major applications in basic research, biotechnology and gene therapy. Initially, exogenous transcriptional control relied on transcription factors with pre-defined target sequences like Gal4 [27] or tetR-fusions [30].This approach strongly contributed to elucidating gene function and creating synthetic gene circuits. However, such engineered TFs are of limited use when it comes to the regulation of endogenous genes, as they require the integration of their target sequence in the intended chromosomal loci. Designer transcription factors with almost unrestricted flexibility of target site selection are a more suitable tool. The first artificial TF to activate an endogenous gene was reported by Beerli *et al*. [40] and consisted of a zinc finger DNA-binding domain and a transcriptional activation domain. The difficulties related to the design and the laborious screening of zinc fingers accelerated the entry of new designer DNA-targeting domains, namely transcription activator-like effectors (TALEs) and the CRISPR/Cas system. The clear one-repeat-to-one DNA base correlation [50] of TALEs makes them an easy to handle tool with only little rules to be observed. Originally acting as transcriptional activators in plants, the applicability of TALEs in eukaryotic cells as nucleases [128], activators [55,59,76], repressors [59,69], recombinases [65] and transposases [66] has been widely reported. A lot of effort was made to optimize TALEs with regard to the length of their N- and C-terminal regions [65,129] and their DNA-targeting RVDs [130]. However, to date no quantitative comparison to existing transcriptional control systems was done. Therefore, this thesis provides the first comparative analysis of TALE-based transcription factors and the intensively studied heterologous transcription factors of the Tet system. The Tet system with its favorable characteristics, including low background activity and high inducibility, is the most widely used transcriptional control system and sets a high standard.

Transcription factors based on the CRISPR/Cas system were included in the comparison as new players in the field of genome engineering. Moreover, the study extended upon comparison by in-depth analysis of different mechanisms of TALE-mediated transcriptional repression.

5.1 TALEs as efficient transcriptional activators

Activation assays were chosen for initial characterization of TALE properties. In contrast to repression assays, they have the benefit of permitting the detection of even moderate activity changes in transient transfection experiments. The detection of repression is always hampered by a preexisting level of reporter expression, making it difficult to monitor small variances. The transcriptional activator tetTALE-AD was constructed to target the 19 bp tet operator sequence and was fused to a C-terminal VP64 activation domain. The TALE length of 18.5 repeats used herein was previously reported to be optimal for high activity [75,131]. This may have contributed to the remarkably high activation levels observed for tetTALE-AD in transient assays as well as on a chromosomal target. In fact, similar activation levels for tetTALE-AD and the Tet system activator tTA were achieved (see Figure 4.1 and 4.3). Moreover, the P_{tet7} luc reporter carried the heptameric *tet*O_7 cassette about 100 bp upstream of the TSS. This provides optimal conditions for TALE-mediated activation, as a clear position dependency of the target sequence relative to the transcriptional start site in reporter assays was shown [117]. Apart from enhancers [132], targeting the region of the proximal promoter up to 200 bp upstream of the TSS is considered best suited to induce transcriptional activation [74,117,133]. The heptameric *tet*O_7 cassette used in the majority of experiments was shown to cause synergistic activation effects when compared to a single target site (see Figure 4.2). This is in line with previous reports of the synergistic action of multiple TALEs binding in one region [75,76,94]. It was suggested that binding of one TALE activator facilitates the binding of the next in a closed chromatin context and the resulting transcription displaces histone H1, otherwise competing with TF binding [134]. Nevertheless, synergy by tetTALE was also observed in transient assays where it presumably resulted from the increased recruitment of coactivators. An inverse correlation between the basal transcription level and the achievable activation level has been described for dCas9-based activators [133]. Assuming this is a general principle, activation assays using a tet-responsive reporter profit from extremely low basal transcription levels.

Using tetTALE constructs targeting both DNA strands, no difference in activation capacity was observed (see Figure 4.4). The orientation of the TALE on DNA seems to be irrelevant, a question that could not be addresses with the tet *trans*-activator, which binds as a dimer. This is in line with a study where TALE activators and repressors targeting a sequence upstream of the promoter were shown to act in a

strand-independent manner [71]. This is a major difference to natural TALEs in plants where the binding site is directional [135]. Using different TALE and reporter mismatch variants aimed at investigating the specificity of tetTALE. Omitting the mandatory first thymine base resulted only in a marginal decrease, comparable to that observed when 5 additional mismatching repeats were added to the N-terminal end of tetTALE (see Figure 4.6). The latter is in accordance with the literature, stating that 5' mismatches have a greater influence on TALE activity than those located more to the 3' end of the target sequence [60,69]. Meckler *et al*. demonstrated that designer TALEs using only a limited range of RVDs are notably less sensitive to substitutions of the first thymine base compared to their natural counterparts [136]. Mutations of the *tet*O that were not located to the outer ends of the target sequence, had a very differential impact on tetTALEs activation ability (see Figure 4.5), demonstrating the high position and context dependence previously described by Zhang *et al*. [55]. Yet, all mismatch experiments in this work were performed transiently and the moderate effects observed might just as well result from overexpression of the reporter.

Although there is still room for improvement as several studies have demonstrated in part marginal activation levels of endogenous genes [75], these results suggest that, under optimal conditions, TALE activators can be a powerful tool that compares to one of the strongest TF known in terms of efficiency. There are a number of conceivable applications for effective TALE-mediated activation, e.g the activation of surrogate genes to compensate for misfunctional genes instead of replacing them [137]. The induction of endogenous genes circumvents the problems arising from the overexpression of only one isoform [138], a factor also worth considering when it comes to reprogramming [73].

5.2 Modes of TALE-mediated repression

Gene repression and gene silencing are often used synonymously to describe transcriptional inhibition. The term silencing is prevalently used to imply long-term epigenetic manifestation of transcriptional inhibition. Repression on the other hand comprises the interaction of transcription factors with the transcription machinery from a distance or close to the gene. The transition between repression and silencing

is fluent, as transcription factors are able to recruit epigenetic cofactors [18], and epigenetic marks themselves attract repressive TFs [139].

In this thesis, the deletion or the exchange of the activation domain with a KRAB silencing domain enabled the discrimination of several modes of TALE-mediated repression. Targeting a heptameric *tet*O sequence located 5' of three mammalian promoters led to total abrogation of expression in a part of the cells (see Figure 4.13 and 4.15). This could be clearly attributed to the presence of the KRAB silencing domain. In line with a previous study using a ZF-KRAB fusion [122], this argues for the possibility to overcome the often described modest repression of endogenous genes [59,67] by carefully determining the optimal target region upstream of the TSS. However, repression capacity differed between the tested mammalian promoters. The strongest repression down to background level was observed with the human EF1α and a clear but only gradual repression with the human Ubiquitin C promoter. Repression was detectable, but less distinctive with the hRosa promoter where the tetTALE-SD binding site was located farthest from the TSS. The KRAB silencing domain is described to act over a long range [18] but differences in promoter architecture and interacting transcription factors may account for the differences. The use of more potent silencing domains like the SID domain [59] or selected combinations of repression domains may further improve repression levels for individual promoters, but have not been tested here.

Apart from *trans*-repression, other potential repression mechanisms were addressed. The obstruction of transcription by a road block mechanism, i.e. the stalling of RNAPII during transcript elongation, was demonstrated with TALEs in prokaryotes with modest repression rates [140]. However, results presented here indicate that TALEs without silencing domain are inefficient in halting the RNAPII when bound to an intragenic region in eukaryotic cells (see Figure 4.19 and 4.20). Contrary to prokaryotic repressors, TALEs wrap around the DNA [53,141] and form a compact protein-DNA interaction. Surprisingly, this seems to be no obstacle to RNAPII. Earlier experiments with zinc finger transcription factors came to the same conclusion [39]. Conceptually similar experiments with catalytically inactive dCas9 resulted in moderate repression [127]. In accordance with data presented here, the effect was improved when a silencing domain was added. The repression in this case is most likely mediated *in trans*, as the KRAB silencing domain is known to work when located 3' of the promoter [142]. For dCas9, Gilbert *et al.* suggest that the distance of

the binding site to the TSS plays a critical role [127]. Uhde-Stone *et al.* demonstrated that TALEs targeting the sense strand of the 5' untranslated region cause a clear reduction of reporter activity, independent of the effector domain [71]. The position effect of the *tet*O_7 cassette on the elongation block is an issue that has not been addressed in this thesis and provides a starting point for further improvement.

Albeit tetTALE was not able to block transcription elongation in the given setting, further analysis evidenced that its binding strength was sufficient to compete with other DNA-binding proteins for identical or overlapping binding sites. The "classic" repressor setting was defined by Jacob and Monod, using the example of the *lac* operon [23]. In this concept, the mutual exclusive binding of the transcription machinery and the *lac* repressor controls the expression of a group of genes. The competition of tetTALE with tTA is a special application of that concept where mutual exclusive binding is not with the transcription machinery but with a transcription activator, which in turn recruits or stimulates the RNAPII. Experiments were performed under stable conditions to minimize distortions due to variations in effector levels that are inherent to transient assays. Using the inducible nature of the Tet system, it was shown that tetTALE could effectively prevent tTA binding or compete with the pre-bound TF (see Figure 4.37 and 4.39). The presence of the silencing domain had only a marginal influence, arguing for a quantitative competition rather than the effect of a few bound repressors interfering with tTA-mediated transcriptional activation. Furthermore, the use of an EGFP reporter in CHO cells revealed that competition can be complete, as total abrogation of reporter activity was observed in a substantial fraction of cells (see Figure 4.44). This supports the notion that the remaining signal detected in the luciferase assay originated from only a few remaining active reporters. The finding was validated in three cell lines using the original and the reverse tet *trans*-activator. Single clone analysis illustrated that repression can be tuned by varying the amount of tetTALE. Overexpression of tetTALE shifted the balance towards repression, whereas in the case of little tetTALE presence tTA was able to prevail (see Figure 4.40). In this regard, it is worth mentioning that the dissociation constant of tetR to the tetO2 operator is in the high picomolar range [143], whereas it is in the low nanomolar range for a TALE with a comparable backbone as used here [60]. This suggests that it is not primarily the strength of individual TALEs but their combinatorial action that renders them effective. The findings reported here substantiate a previous study by Li *et al.* where

overexpression of a shorter *tet*O targeting TALE prevented rtTA-mediated activation [92]. However, in this case *tet*O TALEs were transiently transfected, resulting in imbalanced effector levels. Furthermore, in contrast to experiments reported in this thesis, the TALE constructs reported by Li *et al*. invariably carried effector domains, preventing an exact statement of the underlying repression mechanism [92]. Comparative experiments evidenced that tetTALE-AD and tetTALE-SD were equally effective in inducing activation and repression as the truncated *tet*O targeting TALEs published earlier [92] .

By contrast, another study reported that TALEs were not able to prevent the binding of the transcription factors Klf4 and Nanog at the Nanog enhancer in mouse embryonic stem cells [132]. In this particular case, however, the binding sites of the TFs in question did not overlap with the sequence targeted by the TALE.

Zhang *et al*. achieved up to 30 % repression by fusing two TALE DBDs targeting a region upstream of the TSS [70]. Here, the large fusion protein most likely prevented other factors from binding by steric hindrance. Still, this is a rather modest repression when compared to what was achieved with tetTALE.

In this thesis, the repression by competition was shown to be of more general validity when tetTALE was targeted to two *tet*O sequences proximal to the TATA box of a CMV promoter. The resulting interference with the transcription initiation reduced reporter expression ten-fold in transient experiments. These results question whether the previously reported exceptionally efficient TALE-mediated repression targeted close to the TSS was the result of the silencing domain, as stated by the authors, or resulted from competition with the pre-initiation complex [69].

Apart from general design rules, the encounter with endogenous regulatory factors is considered to have a major influence on the activity of individual TALEs [76]. Results presented here suggest that at least some TALEs are very efficient in binding even to occupied targets. The mutually exclusive binding of different TFs is not an artificial setting but a long since known natural mechanism to exert transcriptional control [144-146]. TALEs ability to offset the effect of other regulatory factors provides a tremendous advantage regarding the regulation of endogenous genes. Expression of an undesirable gene could be greatly reduced or totally suppressed by targeting the binding site of an essential transcription factor. Moreover, targeting the binding site of a common regulator can influence the expression of a whole group of genes.

Furthermore, omitting the silencing domain makes TALEs more suitable for the creation of synthetic circuits as no long term effects are expected [39].

5.3 Epigenetic modifications accompanying transcriptional regulation

The capacity of TALE activators to activate silenced targets like the Oct4 gene without influencing DNA methylation has previously been established [117]. This activation could be increased when TALE activators were used in combination with histone modifying enzymes [117], or when DNA methylation inhibitors were administered globally [74]. Another study indicates that a TALE-VP64 fusion causes activating histone marks without further external stimuli [132]. The silencing of genes is often accompanied by an increase in repressive epigenetic marks like CpG methylation at promoter sites or specific histone modifications like ubiquitylation, methylation or sumoylation [114,147]. As the *trans*-repression with tetTALE-SD was exceptionally effective and the KRAB domain is able to establish repressive epigenetic marks [17], the influence of tetTALE-SD on the methylation of the proximal promoter region of the hEF1α was analyzed in this work. In the setting presented here, no apparent increase in DNA methylation was correlated with reporter repression (see Figure 4.18). Several studies report reversible repression with tetR-KRAB fusion [21,110], arguing for a short-term repression mechanism of KRAB in the context of artificial TFs. On the other hand, reports of TALE-SD causing repressive histone modifications [73] suggest that DNA methylation is not the only epigenetic modulation to consider. It was reported that histone modifications occur earlier than changes in the methylation status [148], as DNA methylation is rather involved in the manifestation and maintenance of an already existing repressive state [149]. Especially for transgenes, methylation was demonstrated to succeed transcriptional inactivation [149]. Together with the fact that conditional tetTALE-SD-mediated repression was completely reversible, this suggests that repression by tetTALE-SD was not manifested by DNA methylation but rather on the chromatin level, if at all. Further studies should focus on the increase of histone modification in highly repressed cell clones.

5.4 Conditional transcription regulation with TALEs

For applications in basic research, conditionality frequently provides a valuable additional level of experimental control. In biotechnology, the high yield manufacturing of recombinant proteins often poses a burden on the producing organism, especially in the case of cytotoxic proteins. In this context it is advantageous to cultivate the cells to a defined growth phase before inducing target protein expression [150]. In gene therapeutic approaches conditional gene expression is often needed to keep the protein amount at physiological levels and limit adverse effects resulting from overproduction. Most of the established systems are ligand-dependent and act on the level of transcription initiation. The requirements include low basal expression combined with dose-dependent inducibility. The ligand needs to be non-toxic and exert no pleiotropic effects [151]. Several such systems have been developed and tested for the conditional expression of TALEs e.g the RheoSwitch [92], the riboswitch [152] and an inducible system based on the abscisic acid receptor [153]. A ligand-independent approach was presented by Konermann *et al*. with light inducible TALE-based transcription factors [153]. Experiments in this thesis, however, focused on two small molecule based systems. The iDimerize system is a tripartite system comprising a responsive reporter construct, a DNA-binding domain fused to FKBP and an effector domain fused to FRAP (see result section 4.5.1 for the identity of the individual components mentioned here). Rapamycin, as the natural heterodimerizer or artificial rapalogs induce proximity of the DBD and the effector domain, resulting in a functional transcriptional regulator [119]. Placing the tetTALE-SD cassette under the control of a heterodimerizer-dependent promoter was expected to result in ligand-dependent repression. In fact, only a small fraction of cells showed a decreased reporter expression in the presence of the ligand, demonstrating an overall low efficiency of regulation (see Figure 4.22). An apparent drawback of the system is that all three components need to be delivered to the cell at sufficient levels. While the activation components were delivered as a polycistronic construct, the inducible tetTALE-SD cassette was located on another plasmid. Even stable integration does not grant equimolar expression of all components. Expression of the activation components was particularly low and only transposon-based stable integration resulted in acceptable levels (data not shown). On the other hand, the inducible tetTALE construct had to be introduced without the help of a transposon, as promoter activity of the 3’ terminal repeat led to

elevated background expression. It was probably the lack of sufficient expression that resulted in the only marginal induced repression. The same system was used lately for conditional transcriptional control with the CRISPR/Cas system [154]. However, in this study the expression of the dCas9 activator was not dependent on rapamycin, but the activator was split and each fragment fused to either FK506 or FKBP. Thereby the system is reduced to two components and activation levels comparable to that of the full-length effector were achieved. Yet, induction for two hours had the same effect as continuous treatment with rapamycin, pointing at very fast induction kinetics but problems with reversibility [154]. This makes the system unsuitable for conditional transcriptional regulation.

The second system assessed for conditional tetTALE expression also relies on the splitting of the effector protein. Here, nonetheless, not two individual proteins are produced but the DNA-binding domain is separated from the effector domain by the introduction of two hormone binding receptors. Conformational change in the presence of the hormone generates a functional transcription factor. This system further simplifies the delivery, as only this single chain switch has to be introduced. Employing this system for the intended analysis of the reversibility of repression proved problematic. The publication first reporting the approach exclusively shows data from transient activation experiments, giving no indication of its behavior after stable integration [94]. The very effective repression by these conditional tetTALE-SDs in the presence of the hormone ponasterone A was not stable after sorting for highly downregulated cell pools (see Figure 4.29). This could in part be attributed to the promoter regulating the expression of the conditional tetTALE, as the CMV promoter is reported to cause a heterogeneous expression pattern and is often silenced [155]. However, using the hEF1α only resulted in an improved but not complete stability of repression (see Figure 4.32). It was rather the cell system and selection method of the repressed cells that had an impact on the longevity of repression. In all cases did the removal of PonA eventuate in a complete reconstitution of initial expression levels, arguing for a reversible repression mechanism.

It was reported that the use of PonA in combination with a ligand for the retinoid X receptor shows synergistic effects and increases fold regulation [156]. This could further improve the regulation and make it applicable in a broader range of cell systems.

5.5 Quantitative comparison of CRISPR/Cas- and TALE-based transcription factors

The Type II CRISPR/Cas9 system originating from *Streptococcus pyogenes* has found rapid use in genome engineering and transcription regulation during the last few years [79,80,126]. Its simplicity, compared to other available bacterial CRISPR/Cas systems, requires only one protein and an engineered sgRNA to target specific DNA sequences. Transcriptional regulation has been established by mutating the Cas9 nuclease, rendering it catalytically inactive and a suitable platform to fuse transcriptional regulatory domains [124]. The comparison to Tet system-derived and tetTALE transcription factors in this work indicated a comparable activation capacity in transient experiments (see Figure 4.50). This is in line with the study from Cheng *et al*. [124], the source of the basic backbone of all constructs used in this thesis. Their comparison of dCas-VP48 to rtTA-mediated activation, using a sgRNA roughly corresponding to sg tetOa, demonstrated high transient activation levels. However, they did not include stable activation experiments, which proved to be severely hampered, presumably by low expression levels of the dCas9 fusions as well as the sgRNA. Several studies, not easy to reconcile, either report a linear correlation between regulation and sgRNA expression [124,127], or no detectable change in efficiency after 10-fold sgRNA dilution [133]. Data presented here indicate that high cellular concentrations of the sgRNA and the dCas9-effector fusions play a vital role and determine activation efficiencies. Expression levels could be increased by using stronger promoters or transposon-based vectors for stable integration.

Activation with the sgRNA sg tetOb, functional in double transient reporter assays, did not work on a chromosomal target. As pointed out by Kearns *et al.*, there is a great variability in activation levels of endogenous genes between different sgRNAs [89]. An underlying reason may be the difference in accessibility of individual loci. In general, dCas9 activators are reported to be less potent in their activation capacity for endogenous genes than TALE-ADs [84,86,117,132]. It has been discussed that the efficacy of dCas9 activators may be compromised when they encounter target sites occupied by endogenous factors [86]. On the other hand, the dCas9/sgRNA complex was reported to prevent the binding of other transcription factors in enhancer regions [132]. To approach this aspect, dCas9 without effector domain was targeted to the site of transcription initiation as previously done for tetTALE. Despite reports to the contrary, where dCas9 bound to the proximal promoter caused

moderate repression in prokaryotic [85] and eukaryotic cells [89], no such effect was observed here. A distinct positive mCherry population, indicative for high dCas9 expression, but no reduction in reporter expression was observed (see Figure 4.52). Hence, unlike the smaller 120 kDA tetTALE, the 160kDA dCas9 protein was not able to prevent transcription initiation under these experimental conditions.
When repression *in trans* with a dCas9-SD fusion was compared to tetTALE-SD performance, dCas9-SD failed to achieve similar repression levels. This is contrary to several publications evidencing the high repression capacity of dCas9-KRAB fusions [117,127]. It has to be noted that experiments here were only performed transiently and might not represent dCas9-SD`s overall repression capacity. Nevertheless, they coincide with results from Zhang *et al.*, indicating stronger repression by TALE-SD than by dCas9-SD in a transient reporter assay [68]. In this thesis, the use of two cell lines revealed a clear cell type dependency. Expanding the investigations to further cell systems would give a more conclusive picture.

The CRISPR/Cas system provides several advantages over TALE-based TFs. The use of sgRNAs obviates the need to assemble a new protein expression cassette for every target. Furthermore, the delivery in multiplexed experimental settings is facilitated. As the turnover rate of RNAs is higher than that of proteins [157], the CRISPR/Cas system is a promising tool for purposes where fast kinetics are required. These benefits have resulted in extensive optimization efforts for Cas9 nuclease applications as well as dCas9-based transcriptional regulators. For example, protein-interacting aptamers were fused to the sgRNA. This can recruit effector domains to the dCas9:sgRNA and was shown to clearly improve activation capacity compared to effector domains directly fused to dCas9 [133]. Further enhancement was achieved when different activation domains were combined, presumably because this better mimics the natural transcription activation process [133,158]. Another way to implement the recruitment of multiple ADs to a single dCas9:sgRNA complex was achieved by using the so-called SunTag system [159]. This system is based on the fusion of an activation domain to a single chain antibody which recognizes a specific epitope linked to dCas9. Thus, up to 24 identical or different regulatory domains can be recruited by a single dCas9:sgRNA complex, causing superior activation. Optimizations concerning the expression of sgRNAs have also been suggested. Introducing the expression of sgRNAs from the H1

RNAPIII promoter broadens the spectrum of possible targets, as it tolerates G and A as the start nucleotide of the sgRNA [160]. Furthermore, sgRNA expression from RNAPII promoters opens up possibilities for conditionality [161].

The implementation of these optimizations was beyond the scope of this thesis but they provide a valuable starting point for further refinement and widespread application. A lot of the optimizations could also be used for TALE transcription factors. Hu *et al.* report that a combined application of TALE- and dCas9-based transcription factors exhibited synergistic action [117]. Combining both systems might be a way to effectively regulate endogenous genes, overcoming the limitations of each individual system.

6 Challenges and outlook

This study provides a systematic evaluation of gene regulation with TAL effectors, which have proven to be remarkably efficient and versatilely applicable. However, there are some aspects that require further investigation to fully exploit the potential of this class of designer transcription factors.

The utilization of TALEs could be extended for example by targeting mitochondrial DNA. Correction of mutations with TALE nucleases was already demonstrated [162,163] but the identification of suitable regulatory domains would enable the repression or overexpression of mitochondrial genes and help unraveling their function.

Most studies assess the functionality of transcriptional regulators over a relatively short period of time. Especially for continuous therapeutic approaches the longevity of control is essential and may differ dependent on the targeted region and its chromosomal context. In this regard, also the off-target activity is an issue of great relevance. A lot of effort was made to analyze the off-target activity of zinc finger nucleases [164], the first generation of custom-defined DNA-binding proteins. The same careful evaluation is necessary for every new class of tailored nuclease or transcription factor. There are indications that TALENs are better in distinguishing between similar sequences than Zinc finger nucleases [165], but mismatch analyses presented in this study make clear that this is most likely dependent on the context and the TALE in question. To reliably predict off-target activity, more data and the development of a robust algorithm are needed. Furthermore, the delivery of TALE effectors to primary cells that are hard to transfect needs optimization. Viral delivery is limited by the size of the TALE expression cassettes and the highly repetitive structure, which is prone to recombination.

Despite these open tasks, the rapid development of tools for precise interventions in the genome and transcriptome of cells via designer DNA-binding proteins opens new opportunities for basic science as well as for clinical applications.

7 Summary

The continuous development of new technologies like ZFP, TALEs and the CRISPR/Cas system to target chromosomal DNA sequences opens more efficient possibilities to edit genomic loci or to modulate the activity of targeted genes. The modular structure of recombinant TALEs allows great flexibility for target site selection. This thesis compares this new technology to one of the best-established heterologous eukaryotic transcriptional control systems, namely the Tet system. Furthermore, it provides particular insights into the different mechanisms of TALE-mediated transcriptional repression.

Creating TALEs targeting the tet operator (tetTALE), a sequence recognized by all common Tet system components, set the stage for a direct comparison in terms of efficacy, efficiency and specificity. When targeted to a region upstream of the proximal promoter sequence, tetTALE activators were demonstrated to be on par with tetR-based transcription factors. This highly efficient regulation was also reflected in *trans*-repression experiments where the repression of three mammalian promoters reached from distinctly decreased to total abrogation of expression. The repression was apparently not epigenetically manifested on the level of DNA methylation.

Additional to silencing domain-mediated repression, TALEs were established as very effective competitive repressors. Taking advantage of the conditional nature of the Tet system, tetTALE was shown to prevail as the dominant transcription factor either when pre-bound and challenged by the tet-transactivator or when encountering a pre-occupied target sequence. This ability to efficiently compete could also be demonstrated for larger protein complexes like the transcription initiation machinery, thus substantiating a broad validity of this repression mechanism.

The clear advantage of the Tet system over the new tailored DNA-targeting tools is its conditionality. However, the introduction of hormone-binding receptors to the tetTALE protein made transcriptional activation and repression dependent on the presence of a small-molecule inducer. Thereby the inducible TALE combines the best of both worlds − the virtual unlimited target site selection and the on-demand transcriptional regulation.

The recently developed CRISPR/Cas-based transcription factors were subjected to a similar, albeit reduced, comparative investigation. Two single guide RNAs, targeting

sequences overlapping with the *tet*O, were used. The analysis revealed a similar transient activation level for TALE- and Cas-based activators. Repression by dCas9-SD, however, was evinced to be inferior to tetTALE-SD, both for *trans*-repression and for a competitive setting.

In summary, the lessons learned from the in-depth analysis of TALE capacity presented here can be used to further optimize transcriptional regulation with this and all future tools for the control of endogenous genes.

8 Zusammenfassung

Die fortlaufende Entwicklung neuer Technologien wie ZFP, TALEs und dem CRISPR/Cas-System zum gezielten Erkennen und Binden chromosomaler DNA-Sequenzen eröffnet immer effizientere Möglichkeiten genomische Loci zu manipulieren oder die Expression von Zielgenen zu beeinflussen. So erlaubt die modulare Struktur von rekombinanten TAL-Effektoren eine hohe Flexibilität bei der Wahl von Zielsequenzen. Diese Arbeit ordnet diese neue Technologie im Vergleich zu dem am besten etablierten System zur heterologen eukaryotischen Transkriptionskontrolle, dem Tet-System, ein. Außerdem bietet sie insbesondere Erkenntnisse über die verschiedenen Mechanismen der TALE-vermittelten Repression.

Das Konstruieren eines TALEs, der genau wie die weitverbreiteten Komponenten des Tet-Systems die Sequenz des tet-Operators erkennt, ermöglicht den direkten Vergleich der Wirksamkeit, Effizienz und Spezifität beider Systeme. Wenn eine Sequenz 5' des proximalen Promoters gebunden wird, ist die Aktivierung durch TALE-Aktivatoren vergleichbar mit der durch tetR-basierte Transkriptionsfaktoren. Diese hoch effiziente Regulation spiegelt sich auch bei der *trans*-Repression wider. Die Repression von drei Promotoren aus Säugerzellen reichte von deutlich verringerter Aktivität bis zur totalen Stilllegung der Expression. Diese Repression wurde nicht durch eine Erhöhung der DNA-Methylierung epigenetisch manifestiert.

Zusätzlich zur Domänen-vermittelten Repression konnte gezeigt werden, dass TAL-Effektoren sehr effizient als kompetitive Repressoren agieren können. Durch die Nutzung der Konditionalität des Tet-Systems konnte gezeigt werden, dass an ihre Zielsequenz gebundene TALEs sich als dominante Transkriptionsfaktoren etablieren, wenn sie mit Tet-Transaktivatoren konfrontiert werden. Gleiches gilt wenn TALEs eine bereits besetzte Bindungsstelle antreffen. Diese Fähigkeit zur effektiven Kompetition konnte auch für größere Proteinkomplexe wie die Transkriptionsinitiations-Maschinerie gezeigt werden, was die Allgemeingültigkeit dieses Repressionsmechanismus verdeutlicht.

Der deutliche Vorteil des Tet-Systems gegenüber den neuen maßgeschneiderten DNA-Bindungsfaktoren ist seine Konditionalität. Das Einbringen von Hormonbindungsdomänen in das tetTALE-Protein machte sowohl die Aktivierung als auch die Repression abhängig von einem „small-molecule inducer". Damit verbindet

der induzierbare TALE die Vorteile beider Systeme – die nahezu unlimitierte Auswahl der Zielsequenz und die Transkriptionsregulation nach Bedarf.
Die kürzlich entwickelten CRISPR/Cas-basierten Transkriptionsfaktoren wurden einer ähnlichen, wenn auch weniger ausführlichen, vergleichenden Untersuchung unterzogen. Es wurden zwei „single guide RNAs“ verwendet, deren Zielsequenzen mit dem tet-Operator überlappen. Die Untersuchung ergab ähnliche transiente Aktivierungslevel für TALE- und Cas-basierte Aktivatoren, wobei letztere jedoch sowohl in der Repression *in trans* als auch in kompetitiven Situationen klar schlechter funktionierten.

Zusammenfassend können die Erkenntnisse, die durch die hier präsentierte eingehende Analyse der Leistungsfähigkeit von TAL-Effektoren gewonnen wurden, für die weitere Optimierung der Transkriptionskontrolle mit diesem wie auch allen zukünftigen Systemen zur transkriptionellen Regulation endogener Gene genutzt werden.

9 References

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10 Abbreviations

°C	degree celsius
AD	Activation domain
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
Cas	CRISPR associated proteins
CHO	chinese hamster ovary
CMV	cytomegalovirus
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
Cu	cupper
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dox	doxycycline
E. coli	*Escherichia coli*
EcR	ecdyson receptor
EDTA	ethylenediaminetetraacetic acid
hEF1α	human elongation factor 1 alpha
EGFP	enhanced green fluorescent protein
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FP	fluorescence protein
h	hour
HA	hemagglutinin
HEK	human embryonic kidney
IPTG	isopropyl-β-D-thiogalactopyranosid
KRAB	krüppel-associated box
LB	lysogeny broth

mA	mili amper
$MgSO_4$	magnesium sulfate
min	minute
ml	mililiter
mM	milimolar
MOI	multiplicity of infection
NaCl	sodium chloride
ng	nanogram
NLS	nuclear localisation signal
nm	nanometer
oligo	oligonucleotide
PB	*PiggyBac*
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
Pen/Strep	penicillin/streptomycin
PNK	polynucleotide kinase
PonA	ponasterone A
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute medium
RXE	retinoid X receptor-α linked to the ecdysone receptor
RXR	retinoid X receptor
SAP	shrimp alkaline phosphatase
SB	*Sleeping Beauty*
SD	silencing domain
SDS	sodium dodecyl sulfate
sgRNA	single guide RNA
TAE	Tris-acetate-EDTA
TALE	transcription activator-like effector
TALEN	transcription activator-like effector nuclease
TBS	tris-buffered saline
TBS-T	TBS-Tween

*tet*O	tet operator
tetR	tet repressor
tracrRNA	*trans*-acting crRNA
tTAs	Tetracycline-induced *trans*-activator
UV	ultra violet
w/o	without
wt	wild type
ZFP	zinc finger protein
µl	microliter
µm	micrometer

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Veröffentlichungen

Veröffentlichungen von Teilen der vorliegenden Dissertation sind von mir wie folgt vorgenommen worden:

Werner, J. and M. Gossen, *Modes of TAL effector-mediated repression.* Nucleic Acids Res, 2014. **42**(21): p. 13061-73.

Erklärung

Ich erkläre an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind.

Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Berlin, den..... .....

Jeannette Werner

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