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Abstract

The expression of a gene is determined by the transcriptional activators and repressors bound to its regulatory regions. It is not clear how these opposing activities are summed to define the degree of silencing of genes within a segment of the eukaryotic chromosome. We show that the general repressor Ssn6 and the silencing protein Sir3 generate inhibitory gradients with similar slopes over a transcribed gene, even though Ssn6 is considered a promoter-specific repressor of single genes, while Sir3 is a regional silencer. When two repression or silencing gradients flank a gene, they have a multiplicative effect on gene expression. A significant amplification of the interacting gradients distinguishes silencing from repression. When a silencing gradient is enhanced, the distance-dependence of the amplification changes and long-range effects are established preferentially. These observations reveal that repression and silencing proteins can attain different tiers in a hierarchy of conserved regulatory modes. The quantitative rules associated with these modes will help to explain the co-expression pattern of adjacent genes in the genome.
Synergy of repression and silencing gradients along the chromosome

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The expression of a gene is determined by the transcriptional activators and repressors bound to its regulatory regions. It is unclear, how these opposing activities are summed up to define the degree of silencing of genes within a segment of the eukaryotic chromosome. We show that the general repressor Ssn6 and the silencing protein Sir3 generate inhibitory gradients with similar slopes over a transcribed gene, even though Ssn6 is considered a promoter specific repressor of single genes, while Sir3 a regional silencer. When two repression or silencing gradients flank a gene, they have a multiplicative effect on gene expression. A significant amplification of the interacting gradients distinguishes silencing from repression. When a silencing gradient is enhanced, the distance-dependence of the amplification changes and long range effects are preferentially established. These observations reveal that repression and silencing proteins can attain different tiers in a hierarchy of conserved regulatory modes. The quantitative rules associated with these modes will help to explain the co-expression pattern of adjacent genes in the genome.

Introduction

Cells differentiate and adapt to environmental changes through the expression of lineage and stimulus-specific genes, and through the repression of inappropriate genes\(^1; 2\). The degree of repression is determined by the interplay between repressors and activators bound to a chromosomal region\(^3; 4; 5\). Insufficient repression of lineage specific genes is a frequent cause of partial or unsuccessful reprogramming of differentiated cells into stem cells\(^6\), which underscores the biotechnological relevance of understanding repression in a quantitative way.
While prokaryotic repression has been studied extensively \textsuperscript{7; 8; 9; 10; 11; 12}, the principles and quantitative rules of eukaryotic transcriptional repression mechanisms are unclear.

The co-repressor Ssn6, along with Tup1, actively represses nearly 3 percent of the genes in the yeast genome \textsuperscript{13}. The general co-repressors are recruited by sequence specific repressors to promoters of genes regulated by signals triggered by DNA damage response, glucose, hypoxia or mating types \textsuperscript{14; 15}. The binding of activators to DNA is typically not impeded by Ssn6 and Tup1. They inhibit transcription of the target gene through multiple mechanisms, such as interaction with the general transcriptional machinery and through recruitment of cofactors that deacetylate histones and alter chromatin structure \textsuperscript{16; 17; 18}.

Silencing was defined as a form of repression that acts at distance and involves the formation of specialized chromatin structures. It stands in contrast to gene-specific repressors, which act at or near the site of transcriptional initiation \textsuperscript{19; 20; 21}. It has been recognized that both inhibitory processes are mediated in part by similar molecular mechanisms, which typically involves nucleosome modifying enzymes. It is unclear how silencing inhibits expression; it affects a step between transcriptional initiation and elongation rather than the recruitment of the transcriptional activator or the polymerase \textsuperscript{22; 23}.

Silencing plays a role in lineage specification of yeast cells. Silencer sequences flank a chromosomal region encompassing a pair of genes, which specify the mating type of the cells. Furthermore, silencing affects genes positioned close to the telomere and exogenous genes inserted into ribosomal DNA arrays. Silencing in these regions is less robust than that at the mating-type loci \textsuperscript{20; 24; 25}. The silencers recruit the Sir2, Sir3 and Sir4 silencing proteins. Sir2 deacetylates histones, providing
high affinity docking sites for Sir3. Sir3 and Sir4 bind to each other in vitro with a high degree of cooperativity \(^{26}\). The subsequent binding of the Sir2-Sir3-Sir4 protein complex is thought to enable their spreading along chromatin by “sequential deacetylation” \(^{21; 27; 28}\). Other mechanisms, such as looping of DNA, have been also invoked to explain the action of Sir proteins at distance \(^{29}\).

**Results**

**Repression by Ssn6 when it is recruited upstream or downstream of a gene**

We explored the steady-state behavior of Ssn6 mediated repression in the yeast *S. cerevisiae* with dual-control gene constructs, where the relative position of activator and repressor binding sites was varied (Fig. 1a). The activator GEV binds to a modified *GAL1* promoter, \(P_{GAL1NR}\), which lacks the Mig1p recognition sites \(^{30}\). GEV is composed of a Gal4p DNA binding domain, an estradiol receptor domain, and the transcriptional activation domain, VP16 \(^{31}\). Incremental induction of gene expression by estradiol generated a graded response, as measured by the fluorescence of GFP in a cell population (Fig. 1b). Two tetR binding sites (*tet* operators) were placed upstream of \(P_{GAL1NR}\) to serve as docking sites for the tetR-Ssn6 fusion protein. Doxycycline induces the dissociation of tetR from the DNA. In this way, the intensity of transcriptional activation and repression can be adjusted independently. Increasing the doxycycline concentration results in a graded derepression of gene expression in a cell population (Fig. 1c), in steady-state conditions (Fig. S1).

The efficiency of repression was examined over a broad range of transcription rates induced by estradiol. TetR-Ssn6 reduced the expression only 2-3 times at maximal gene expression but up to 50 times at lower activation of transcription (Fig. 1c).
1d). Repression was slightly stronger when seven tet operators were placed upstream of the promoter (Fig. S5).

In contrast to higher eukaryotes, yeast transcriptional activators and repressors exert their influence within the promoter and over short distances, typically less than 0.5 to 1 kb. We explored the effect of repressor binding to sites considered to be outside of the regulatory region of the gene. For this purpose, four tet operators were inserted downstream of the reporter gene GFP, at a distance of 1 kb from the promoter (Fig. 1a). The reporter construct and the tet operators were flanked by well-defined transcriptional terminators. No inhibition was observed at maximal gene expression. Surprisingly, fold inhibition increased up to five at low intensity of gene activation (Fig. 1d). Binding of the tetR’-VP16 activator to these sites neither repressed nor activated transcription (Fig. 2a).

When fold inhibition-1 was plotted against normalized gene expression (see Materials and methods), measured in non-repressive conditions, similar profiles were seen for both the upstream (proximal) and downstream (distant) repression constructs (Fig. 1e). This similarity was confirmed by fitting an inhibition function to the experimental data. The inhibition function incorporates two forms of repression mechanisms (see below). When the inhibition function, fitted to the upstream construct, was shifted downwards, it matched the data points obtained for the downstream construct (Fig. 1e). Thus, repression by Ssn6 from the upstream and downstream sites is mediated by kinetically similar mechanisms, only the respective repression strengths differ (see Materials and Methods for the calculation of repression / inhibition strength).

**Competitive and supercompetitive forms of repression**
The increase in fold inhibition-1 with decreasing gene activation suggests some form of competition. Competition can occur between the effectors of activators and repressors during the recruitment of the transcriptional machinery (see supporting text). When the occupancy of Ssn6 binding sites was reduced by doxycycline, the repression profiles were transformed nonlinearly; the inhibition curves flattened out (Fig. 1f). Fold-inhibition-1 doubled at most, when gene activation was reduced from half-maximal to an arbitrarily low detectable value. This behavior is typical of competitive inhibition (Fig. 1g blue lines). 35

On the other hand, the value of the inhibition function increased more than twice in the range below the half-maximal expression, when the occupancy of repressor binding sites was high (doxycycline = 0.015 μM, Fig. 1f). We termed this behavior supercompetitive inhibition (Fig. 1g, black dotted lines). This behavior is compatible with models that include a controlled transition between two states of a gene: one is permissive while the other is non-permissive for an initiated transcription (see Supporting text). The transition between these two states is catalyzed by the mediators of the activator and repressor.

Each inhibition function was fit to experimental data by adjusting the contributions of competitive and supercompetitive repression. The contribution of supercompetitive inhibition is more pronounced at high occupancy of repressor binding sites (Fig. 1f).

It is interesting that the contribution of an inhibitory mode changes gradually with the binding of the repressor. This may reflect that the proportion of mediators recruited by Ssn6 varies as a function of Ssn6 binding site occupancy. Such a relation has been described for transcriptional activators 36.
Inhibitory gradients of similar slopes are generated by Ssn6 and Sir3

Constructs were designed to increase the distance between the promoter and tet operators by lengthening the reporter gene (Fig. 2b). Duplicating the GFP sequence in a tandem array reduced the strength of inhibition more than twice (Fig. 2b, c). When the reporter was lengthened to 4.2 kb by inserting a lacZ sequence, repression diminished below the detection limit.

The unexpected effect of Ssn6 at distance prompted us to compare it with Sir3, which is known to spread along the chromosome \(^{37, 38}\). When the above gene expression systems were regulated by the tetR-Sir3 fusion protein, the distance-dependent decrease of silencing strength was similar to that of repression (Fig. 2c, d). This indicates that both repression and silencing generate comparable single inhibitory gradients.

Long range interactions can arise through looping of DNA, as well. Looping was reported to occur between promoters and terminators during transcription \(^{39}\). To test whether the terminator mediated looping accounts for the inhibitory effects of Ssn6 from the downstream sites, a terminator was inserted between a GFP and YFP sequence. In the resulting GFP-T-YFP construct, the tet operators were placed at a distance of 1 kb from the transcriptional termination site of the GFP reporter gene (Fig. 2a). This separation is expected to reduce the efficiency of looping mediated repression. We did not observe a reduction of inhibition strength at the GFP-T-YFP construct in comparison to \([\text{GFP}]_2\) (Fig. 2e, f).

To assess the features of the inhibitory gradients in more detail, constructs were designed, in which either the reporter gene was shortened or the sequence separating the upstream tet operators and the promoter was prolonged (Fig. 3). Silencing nucleated upstream of the promoter was weak. It displayed an inhibition
function comparable to that produced by low occupancy repression sites (Figs. 3a, b, S3). Inhibition by Ssn6 and Sir3 declined more precipitously upstream than downstream of the transcriptional initiation site (Fig. 3c).

The slope of a gradient is a useful measure of action at distance because it is independent of the absolute intensity of the effect. While the action at distance has been considered to be a distinguishing feature of silencing, the slopes of single inhibitory gradients generated by Ssn6 and Sir3 are remarkably similar, both upstream and downstream of the transcription initiation site (Fig. 3c).

**Quantitative properties of silencing by Sir3**

Although the spreading of the Sir2-Sir3-Sir4 protein complex is a phenomenon common to silencing at the telomere and mating-type loci, the molecular mechanisms of the recruitment / nucleation of silencing varies at these loci.\(^{20}\) We explored if the Sir3-nucleated silencing reproduces characteristic features of the endogenous silencing: the distance dependence, cooperation of silencers and variegated expression.

Inhibition of gene expression by telomeric silencing drops around 10 times over a distance of 1 kb\(^{40}\), which is comparable to the slope of the gradients shown in Fig. 3c.

The silencers flanking the mating type loci cooperate. A gene flanked by the HML E and I silencers is repressed up to 50 times, even though individually they are not capable of repressing the gene.\(^{41}\) The cooperativity of Sir3 nucleated silencing was explored by a construct, in which the *tet* operators flanked the expression unit. The fold inhibition-1 of the dual silencing construct reached a value of around 10 and up to 30 in some constructs, when the upstream and downstream sites individually
reached a value of around one (Fig. 4a, b, and see Interaction of silencing gradients below). Furthermore, we examined the interaction of silencing nucleated by Sir3 and the HML E-element. The E element alone did not inhibit expression (Fig. 4c). The ratio of silencing by the combined tetO-GFP-E silencer construct to the silencing by the parent constructs was similar to that nucleated by Sir3 only (Fig. 4d).

Variegated expression has been observed for genes flanked by the mating-type loci silencers, in \textit{sir1} cells \textsuperscript{42; 43}. The dual silencing construct also displayed a broad bimodal (variegated) expression in a cell population, at intermediate estradiol concentrations (Fig. 4e). The above results show that the distance dependence, the cooperative action of silencers and variegated expression at the native silenced genomic loci are very similar to that of generated by Sir3 nucleated silencing.

Chromosomal position effect is also typical of silencing. Among the examined loci, silencing was the strongest at the \textit{YFR054c} locus, positioned 11 kb from the telomere, 2 - 3 times higher than most of the other loci examined (Fig. 4f). Loci closer to the telomere typically display an even higher degree of silencing \textsuperscript{40}. Nevertheless, silencing at the \textit{IRC7} locus, which is 6 kb more telomere-proximal than the \textit{YFR054c} locus, was comparable to that at the \textit{FIG1} locus, located 400 kb away from the telomere (Fig. 4f). This may reflect the discontinuous nature of silencing, which is frequently caused by insulators encountered at telomeres \textsuperscript{44; 45}. A genome wide analysis revealed that hypoacetylated subtelomeric regions are enriched in Ssn6-Tup1 regulated genes \textsuperscript{46}. The \textit{BAT2} and \textit{DAN1} loci are found in the subtelomeric domain of chromosome X. A comparison of downstream repression constructs revealed that the \textit{DAN1} locus imparts around 1.6 times stronger repression relative to the other loci (Fig. 4g). Thus, silencing can be enhanced at positions close to the telomere; while repression in hypoacetylated subtelomeric domains.
Interaction of repression gradients

Since two silencers show a strong cooperative interaction, we tested if two repression gradients cooperate by combining the upstream and downstream repression sites into dual repression constructs (Fig. 5a). When upstream and downstream constructs with nearly equal inhibition were combined, the resulting dual construct displayed 3.5 times higher inhibition strength in comparison to the parent constructs (Fig. 5b).

When two gradients affect the same component or parallel pathways in the transcriptional repression process, they have an additive effect. Alternatively, when the two gradients affect different components in subsequent stages of a process, they have a multiplicative effect on gene expression. When upstream and downstream constructs with equal inhibition strengths are combined, the additive and the multiplicative mechanisms predict similar inhibition functions (Fig. 5c). When constructs with dissimilar (weak and strong) inhibition are combined, the two mechanisms become distinguishable. The additive mechanism implies nearly identical inhibition functions for the single strong and the dual repression constructs (Fig. 5c, upper dashed lines). However, these two functions diverge as the activation of gene expression is reduced, assuming a multiplicative mechanism (Fig. 5c, upper dotted lines).

The multiplicative mechanism fitted the experimental data more faithfully, when constructs with dissimilar upstream and downstream inhibition strengths were combined (Fig. 5d). This behavior was not affected by changing the chromosomal position of the construct (Fig. 5e). Therefore, the function of the dual repression constructs was approximated by the product of the inhibition functions of the single
repression constructs, which was multiplied by a constant to account for a small degree of amplification.

This amplification was seen with an upstream construct incorporating a longer spacer sequence between the \textit{tet} operators and the $P_{GAL1NR}$, which does not display a detectable inhibition per se (Fig. 5f). When it was combined into a dual repression construct, a 1.6 times stronger inhibition was observed in comparison to the downstream repression construct (Fig. 5f).

The multiplicative effect implies that mutations in some of the mediators of Ssn6 would affect the upstream and downstream repression constructs asymmetrically. When effectors of Ssn6, such as the Hda1 histone deacetylase or the component of the mediator, Srb10, are mutated individually, a variable, usually small degree of derepression ensues in a subset of target genes \cite{13, 47, 48, 49}. \textit{HDA1} disruption caused a nearly twofold increase in the absolute expression level of the reporter gene but the relative inhibition strength was unaffected (Fig. S6). \textit{SRB10} disruption led to a twofold reduction in the inhibition strength in the downstream repression construct (Figs. 5g, S7), while only a 1.2 fold reduction was observed for the upstream repression construct. The asymmetric effect of \textit{SRB10} disruption underscores the multiplicative mechanism at the molecular level.

We also examined if repression gradients could affect expression of endogenous genes. Expression of \textit{FIG1} was reduced 2 times when tetR-Ssn6 bound to sites downstream of the 1 kb long \textit{FIG1} gene (Table S4). Genome-wide studies suggest that chromatin modifications, a hallmark of repression and silencing, have a major impact on the correlation between the expression of adjacent genes \cite{50}. Given the widespread role of the Ssn6 / Tup1 in repression and their evolutionary
conservation, it is plausible that repression gradients contribute to the determination of co-expression patterns of adjacent genes.

**Amplification of the silencing gradients**

The inhibition strength of the dual silencing construct at the *FIG1* locus was 6.6 times higher than expected from a multiplicative effect (Fig. 4a). The inhibition functions of dual silencing constructs correspond to the product of amplified inhibition function of the downstream and the inhibition function of the upstream constructs. A strong, approximately 5 fold, amplification was also observed in the dual silencing construct where the upstream sites alone did not inhibit expression (Fig. 4b). While the amplification of interacting repression gradients varied between 1.3 and 1.6, the above values for silencing are considerably higher.

Amplification can arise when a inhibitory gradient enhances the nucleation and/or the spreading of a neighboring gradient (Fig. S8). If one of the single gradients is strengthened, the neighboring gradient would spread more efficiently, provided the effect of Sir3 on transcription and spreading correlate. This scenario can be tested by plotting the degree of amplification as a function of distance between the *tet* operators that flank the gene expression unit. A more efficient spreading would entail a less steep decline in the amplification as the distance is increased (Fig. S8). We took advantage of the observation that the downstream gradient is 2.3 times stronger at the *YFR054C* locus in comparison to the *FIG1* locus.

The distance dependence of the amplification was tested using the dual inhibition constructs with the GFP, tandem GFP, GFP-T-YFP and GFP-T-lacZ reporter genes (Fig. 6a), at the *FIG1* locus. The silencing strength got reduced more than twice, when the GFP sequence was duplicated (Fig. 6b). Silencing at the 4 kb
long GFP-T lacZ reporter was not stronger than the silencing at the corresponding upstream constructs (Fig. 6b, c).

Next, we examined the synergy of gradients at the YFR054C locus (Fig. 6d). The dual silencing construct containing the GFP-T lacZ reporter showed a considerable silencing at the YFR054C locus; at least five times more than expected from its behavior at FIG1 locus (Fig. 6d). This indicates that gradients interacting over long distances are preferentially amplified.

When amplification was plotted as a function of distance, it became evident that the decline of amplification was less steep at the YFR054C locus, in comparison to the FIG1 locus (Fig. 6e). This confirms that silencing has the ability to increase its effect at distance through the amplification of interacting gradients.

A more efficient spreading of the Sir proteins between two nucleation sites can be achieved by a stronger deacetylation through Sir2. Furthermore, increasing Sir3 concentrations induce an increasing compaction of the chromatin in vitro. Compaction can create contacts between non-neighboring nucleosomes, which shortens the distance between the two gradients. Consequently, the spreading of the silencing factors is facilitated.

**Discussion**

We employed dual control gene expression systems to compose an inhibition function that adequately describes the effect of the Ssn6 repressor and Sir3 silencing proteins on gene expression. The inhibition function, which includes competitive and supercompetitive forms of repression, was then used to characterize the distance dependent action and the synergy. It was surprising that transcriptional inhibition by Ssn6 and Sir3 can be described by a gradient with nearly identical slopes, because
silencing was distinguished by its ability to inhibit transcription at distance; and Sir proteins were shown to spread along the chromosome. However, when two gradients interact, Ssn6 and Sir3 behaved differently.

The inhibition of gene expression by Ssn6 is adequately described by the product of the upstream and downstream repression functions. The multiplicative interaction arises because the components mediating the repression are asymmetrically affected by the upstream and downstream gradients (Figs. 5g, 7a). The repression gradients undergo a small, less than twofold amplification during the interaction.

The term silencer was coined for a sequence at the mating-type locus that represses gene expression at a distance\textsuperscript{19}. Our results refine the definition of silencing regarding its effect on transcription. While both repressor and silencing proteins can generate transcriptional inhibitory gradients of equal slopes, only silencing gradients, which flank a gene, can undergo significant amplification during their interaction. Strengthening of a single gradient is translated into a more gradual decline of transcriptional inhibition along the chromosome when it interacts with a neighboring gradient (Figs. 6e, 7b). Thus, long-range effects specific to silencing proteins do not appear at the level of isolated gradients, but arise due to the synergistic interaction of the single gradients. The advantage of dispersed nucleation sites that flank a gene over clustered ones is also evidenced by comparing constructs where the total number of operators is nearly equal. For example, Sir3 binding to two upstream and four downstream operators produced nearly two times stronger silencing than binding to seven upstream operators (Figs. 4a, S5). The understanding of the formation and interaction of gradients is important to explain the correlations between the expression of adjacent genes, in terms of the steady-state level and fluctuations\textsuperscript{52, 53}. 
Ssn6 and Sir3 can be conceived as two points in a continuum of repression modes. Even though amplification is typical of silencing, residual amplification is observed also for Ssn6. The continual nature of repression modes is also supported by observations that a single mutation can convert a repressor into a protein whose chromatin modifying properties are reminiscent of silencing proteins \(^{54}\). Using the outlined approach, different repressor / silencing proteins can be classified functionally, according to their kinetic form of inhibition, the slopes of their inhibitory gradients, the additive or multiplicative nature of interaction, and the magnitude and linearity of amplification during the interaction of the gradients. Such a characterization is essential to design biological systems, and to employ them for biotechnological purposes, such as enhancement of cellular differentiation through regulation of gene expression in a chromosomal segment \(^{6}\).
Materials and Methods

Yeast strains and growth conditions

All strains are cogenic with the S288C derivatives, BY4741 and BY4742. Yeast strains and construction of plasmids with the reporter genes are described in Tables S5-8. tetR-Ssn6 is obtained from pCM242. Integration of genetic constructs into various chromosomal loci and copy numbers were verified by Southern-blot. All reporter constructs used for silencing have a single-copy integration to avoid long-range interactions. For repression, multiple copy constructs were also used to broaden the range of detectable expression levels. Cells were grown for 6 hours after induction in minimal media supplemented with 2% glucose, starting at a cell density of OD_{600} = 0.05.

Southern-blotting

Yeast genomic DNA was digested with restriction endonucleases and transferred to Hybond-N+ membrane (Amersham) after electrophoresis. DIG-labeled DNA probes were generated with DIG-High Prime (Roche) according to the random primed labeling technique. Anti-digoxigenin-alkaline phosphatase was used for detection and the chemiluminescent signal was recorded with a CCD camera (Fig. S9).

Quantification of RNA levels

Total RNA was isolated by RiboPure Yeast Kit (Ambion). cDNA synthesis was primed with a mix of oligo-dT and random primers using QuantiTect (Qiagen) and was quantified by real-time quantitative PCR. ACT1 was used as an internal standard.
**β-galactosidase assay**

Cells were broken with liquid nitrogen by repeated freeze / thaw cycles. The β-galactosidase activity was measured by colorimetry using CPRG as a substrate.

**Flow cytometry**

GFP expression was measured with flow cytometry using a gating in the side and forward scatter plots to select 5-15% of the total cell population. Expression equals \( \frac{F - C}{C} \). \( F \) is the fluorescence of cells expressing GFP at the applied concentration of estradiol (\( e \)) and doxycycline (\( d \)), whereas \( C \) is the background fluorescence when \( d = 0 \), \( e = 0 \). Normalized expression equals \( \frac{F}{F_{\text{max}}} \). \( F_{\text{max}} \) is the expression at \( e = 200 \text{ nM} \) and \( d = 2 \mu\text{M} \). Fold inhibition-1 is identical to percent inhibition divided by 100. The characteristic features of inhibition are better displayed with fold inhibition-1 than with fold inhibition, when inhibition is weak. The coefficient of variation of flowcytometric measurements is less than 5%. The Standard deviation of inhibition strengths or that of their ratios was calculated from experiments performed on different days.

**Fitting of the inhibition function**

The models of prokaryotic gene regulation are typically based on statistical weights of promoter configurations \(^8\ 9\). Repression by Ssn6 is indirect and includes multiple mechanisms. Hence, the repressor functions, \( f_1(R) \) and \( f_2(R) \), were incorporated into functions for competitive binding, and antagonistic regulation of permissive state, respectively. The values of \( f_1(R) \) and \( f_2(R) \) were fitted to individual experiments while the following parameters were fixed: \( K_A = 1.43 \) and \( \alpha = 0.011 \) in SEq. 5. The correlation between \( f_1(R) \) and \( f_2(R) \) was often higher than 0.99 during nonlinear
repression, when supercompetitive inhibition was present. In this case, \( f_1(R) \) was fixed at a value, obtained at a the lowest doxycycline concentration without significant supercompetitive inhibition.

**Calculation of synergy**

Inhibition functions were fitted for the upstream and downstream constructs. The mean inhibition (repression or silencing) strength, \( I \), is defined as the definite integral of the inhibition functions calculated on the interval \( NE = [0.06, 0.6] \) to represent the regulatory range around the half-maximal expression. For multiplication, the inhibition function and not the inhibition function-1 was used. For simplicity, the amplification factor, \( a \), was multiplied with the inhibition function-1 of the downstream construct and was fit using the experimental data obtained from the dual inhibition construct.

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References


Fig. 1. Effect of upstream and downstream repression sites on gene expression.

(a) Transcriptional terminators (black arrowheads) serve to prevent transcription from interfering with binding to the tet operators. (b) Single cell distribution of fluorescence (PRY438) was measured by flow cytometry as the estradiol concentration was varied (0, 2, 4.5, 15, 110 nM from left to right) in the presence of 2 μM doxycycline (non-repressive condition). (c) Single cell distribution of fluorescence (PRY438) as doxycycline was varied (0, 0.013, 0.033, 0.084, 2 μM from left to right) in the presence of 6.7 nM estradiol. (d) Filled and empty symbols denote expression in non-repressive (2 μM doxycycline) and repressive (no doxycycline) conditions (PRY430, 432). (e) Fold inhibition was calculated from data shown in (D). The ratio of the inhibition strengths of the downstream and upstream constructs is 0.078. The mean and standard deviation of this factor was 0.062 ± 0.013 from three independent experiments. (f) Repression at the tetO2-GFP construct (PRY432). Contributions of competitive, \( f_1(R) \), and supercompetitive, \( f_2(R) \), forms of repression to the inhibition functions were obtained from fitting SEq. 5 to experimental data. \( f_1(R) = 3.98, 5.76 \) and 5.76 fixed; \( f_2(R) = 0, 0.005 \) and 0.14, when doxycycline concentration was adjusted to 0.062, 0.039 and 0.015 μM, respectively. (g) Blue lines represent inhibition functions by solving SEq. 5 for three different intensities of competitive inhibition \( f_1(R) = 0.4, 1.6 \) and 6.4 in the absence of supercompetitive inhibition \( (f_2(R) = 0) \). In contrast, \( f_1(R) = 0 \) for the black dashed lines, while the value of \( f_2(R) \) was set to 0.1, 0.4 and 1.6.
Fig. 2. Distance dependence of repression and silencing from downstream sites.
(a) tetR-VP16 has no effect on transcription from a downstream site. Empty and filled symbols denote expression at 0 and 2 μM doxycycline (PRY368). Expression induced by rtTA at a tetO7-GFP construct is shown in Figure S2. (b) Reporter constructs with increasing length are regulated by tetR-Ssn6 (PRY418, 419, 420, 421) or with tetR-Sir3 (PRY364, 365, 366, 367). β-Galactosidase activity was not detected in the strains carrying the GFP-T-lacZ construct (Table S3). (c and d) Expression under non-repressive (filled circles) and repressive conditions (empty circles). (e) The repression strength relative to GFP is 0.43 (0.33 ± 0.13) and 0.59 for the [GFP]_2 and GFP-T-YFP constructs, respectively. (f) The silencing strength relative to GFP is 0.36 (0.36 ± 0.05) and 0.71 for the [GFP]_2 and GFP-T-YFP constructs, respectively.

Fig. 3. Reconstruction of the upstream and downstream gradients.
(a and b) The tetO2 operators were adjacent to P_{GAL1NR} or separated from it by T_{GAL7}, T_{GAL7} Spacer B-300, T_{GAL7} Spacer B-500 sequences. The spacer B sequence encompasses part of the open reading frame and the transcriptional terminator region of the RPN12 gene. Constructs were regulated by tetR-Ssn6 (PRY 438, 475.4, 483.1, 450) (a); and by tetR-Sir3 (PRY351, 474.4) (b). (c) The mean inhibition strengths (see Materials and methods) of the constructs shown in Figures 2c, 2d, 3a, 3b, S4. The ratios of inhibition strengths at the lacZ(1-150) and lacZ(1-450) constructs were used to define the strength relative to GFP. A mean inhibition strength of 0.075 corresponds to the detection limit. Position denotes the distance between the TATA box and the tet operators.
Fig. 4. Characteristic features of silencing nucleated by Sir3

(a) I (mean silencing strength) = 4.3 was for tetO2-GFP-tetO4 at the FIG1 locus (PRY379). I = 0.65 for the product of the inhibition functions (gray dotted lines) obtained for the parent constructs (PRY371, 378). (b) Silencing at the YFR054c locus, when the upstream operators are separated by the spacer B-500 from P\textsubscript{GAL1NR} (PRY364, 423B, 422B). I = 4.15 and 0.8, for the dual construct and the product function, respectively. (c) Expression of tetO7-GFP (PRY370) under non-repressive (filled circle) and repressive (empty circle) conditions. The expression of the tetO7-GFP-E-element (PRY533.10) in non-repressive condition (empty triangles) is inhibited only by the E-element. The degree of inhibition by the E-element is very low (< 15%, compare filled circles and empty triangles). (d) Silencing strength at the tetO7-GFP-tetO4 (PRY372) and tetO7-GFP-E-element (PRY533.10) constructs is 4.6 and 5.2 times higher than the product of inhibition functions of the parent constructs. The upstream parent for both constructs is tetO7-GFP (PRY370). (e) Single cell distribution of gene expression in the tetO2-GFP-tetO4 construct (PRY355) as the estradiol concentration was varied in repressive condition. (f) P\textsubscript{GAL1NR}-GFP-tetO4 constructs were inserted into the indicated chromosomal loci, in strains with tetR-Sir3 (PRY342, 364, 371, 474.4). The FIG1 locus displays silencing by a factor of 0.40 ± 0.09 less than the YFR054c locus. Empty circles stand for P\textsubscript{GAL1NR}-[GFP]\textsubscript{2}-tetO4 at the FIG1 locus (PRY453). (g) Strains constructed as in (f) but with tetR-Ssn6 (PRY379A, 386, 387, 457, 496.7) The FIG1 locus displays repression by a factor of 0.62 ± 0.04 less than the DANI locus.
**Fig. 5.** Combined effect of upstream and downstream repression sites on gene expression.

(a and b) The inhibition strength at the tetO2-T\textsubscript{GAL7}-GFP-tetO4 construct (PRY478.1) was $I = 2.3$, while for the upstream and downstream constructs (PRY475.4, 418) $I = 0.57$ and 0.65, respectively. The black line is the constant multiple ($a = 1.3$) of the product of the upstream (blue) and downstream (red) inhibition functions. (c) Gray dashed and dotted lines denote the sum and product of two inhibition functions, respectively. Black lines are constant multiples of the gray lines. Interactions were calculated between two red (identical inhibition) curves and between the red and blue (dissimilar inhibition) curves. (d and e) tetO2-GFP-tetO4 and its parent constructs were inserted at the \textit{YFR054c} locus (PRY418, 438, 355.1; $a = 1.4$) (d) and the \textit{FIG1} locus (PRY389, 390, 391; $a = 1.48$) (e). (f) The amplification factor for the dual inhibition construct containing tetO2- T\textsubscript{GAL7} Spacer B-500 is $a = 1.59$ (PRY418, 451B, 450B). (g) Empty symbols stand for the srb10 strains containing the tetO2-GFP (PRY393, 441) and GFP-tetO4 constructs (PRY418, 445). The mean relative difference of repression strength between the srb10 and WT cells are $-0.12 \pm 0.15$ and $0.53 \pm 0.14$ for the respective constructs.

**Fig. 6.** Amplification of silencing gradients.

(a) Dual inhibition constructs with reporter genes of varying lengths. (b and c) Dual silencing constructs at the \textit{FIG1} locus (PRY379, 434, 435, 436) (B). The upstream silencing constructs with the respective reporter genes (tetO2-GFP, tetO2-[GFP]$_2$, tetO2-GFP-T-lacZ; in the strains PRY378, 461, 462) are denoted by the corresponding empty symbols in (c). They behave similarly (scattered around the purple line). Functions for the downstream constructs (black, blue and red lines) are
shown in (c). (d) The dual silencing constructs (PRY355, 501.5, 499; YSSD225) and the tetO2-GFP upstream silencing constructs were integrated into the YFR054C locus (PRY351). (f) Amplification factors (for the strains shown in b, d) were calculated relative to the GFP-tetO4 at the FIG1 or YFR054C locus. The mean and standard deviation of the relative amplification factors were calculated from three independent experiments for the GFP, [GFP]_2 and GFP-T-YFP and GFP-T-lacZ, respectively.

**Fig.7.** Hierarchy of regulatory modes in transcriptional repression and silencing.

(a) Single upstream and downstream gradients (blue and red dashed lines) undergo amplification during interaction (full lines). (b) The degree of inhibition is shown for a given intensity of gene activation when the position of the upstream operator is fixed (blue dotted line) and the distance of the downstream operator is varied (red dotted line). The multiplicative inhibition for the dual repression construct is calculated assuming no amplification (gray line) or amplification caused by moderate and strong enhancement of spreading (blue and green full lines).
Figure 1

(a) tetR - Ssn6

(b) Cell count

(c) Fluorescence [AU]

(d) Expression [AU]

(e) Fold inhibition - 1

(f) Doxycycline [μM]

(g) Fold inhibition - 1
Fig. 2
Fig. 3
Fig. 4

(a) (b) (c) (d) (e) (f) (g)

- Ssn6
- YFR054c
- DAN1
- BAT2
- IRC7
- tetO-GFP-tetO
- GFP - tetO
- tetO-GFP-E
- tetO-GFP

- Estradiol [nM]
- Normalized expression
- Fold inhibition – 1
- Expression [AU]
- Fluorescence [AU]
- Cell count

- YFR054c
- FIG1
- DAN1
- BAT2
- IRC7
Fig. 5
Fig. 6
Multiplicative interaction

Long-range -
Ssn6
Sir3

Short-range -
No amplification

(a) (b)

Fig. 7