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Protein-mediated chromosome pairing of repetitive arrays

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Abstract

Chromosomally-integrated arrays of *lacO* and *tetO* operator sites visualized by LacI and TetR repressor proteins fused with GFP (or other fluorescent proteins) are widely used to monitor the behavior of chromosomal loci in various systems. Yet, insertion of such arrays and expression of the corresponding proteins is known to perturb genomic architecture. In several cases, juxtaposition of such arrays located on different chromosomes has been inferred to reflect pairing of the corresponding loci. Here, we report that a version of TetR-GFP mutated to disrupt GFP dimerization (TetR-A206KGFP or "TetR-kGFP"), abolishes pairing of *tetO*-arrays *in vivo* and brings spatial proximity of chromosomal loci marked with those arrays back to the wild type level. These data argue that pairing of arrays is caused by GFP dimerization and thus presents an example of protein-assisted interaction in chromosomes. Arrays marked with another protein, TetR-tdTomato, which has a propensity to form intra-molecular dimers instead of inter-molecular dimers, also display reduced level of pairing, supporting this idea. TetR-kGFP provides an improved system for studying chromosomal loci with a low pairing background.

INTRODUCTION

Three-dimensional organization of the genome is important for many biological processes including maintenance of genomic integrity[1; 2; 3; 4] and gene expression[5; 6; 7; 8]. Studies in bacteria[1], fungi[9; 10], insects[6] and mammals[3; 7; 11; 12] revealed complex and dynamic nature of genomic architecture. A popular approach to studying three-dimensional organization of the genome is FROS (Fluorescent Repressor-Operator System), which involves tagging chromosomal loci with arrays of directly repeated *lacO* or *tetO* operator sites[13; 14]. Expression of the cognate binding proteins from *E. coli*, lactose repressor (LacI) or tetracycline repressor (TetR) fused with a fluorescent protein (most commonly, GFP) allows direct visualization of the arrays by fluorescence microscopy. These two FROS systems (*lacO*-array/GFP-LacI and *tetO*-array/TetR-GFP) have been extensively used to study chromosome dynamics in bacteria[15; 16], fungi[10; 13; 14; 17; 18; 19; 20; 21], plants[22; 23], nematode[24] and mammalian cells[25]. Even though this methodology is a powerful tool to visualize genomic loci, insertion of arrays with concomitant expression of repressor-GFP fusion proteins can perturb 3D-structure of the genome, thus complicating the analysis.

Molecules of GFP are capable of dimerizing in an anti-parallel manner, by way of hydrophobic interactions on the dimerization surface[26; 27]. Introduction of a single point mutation in one of the hydrophobic amino acids on the dimerization interface drastically

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reduces protein's ability to form dimers. A number of such mutations have been described including A206K, which replaces hydrophobic Alanine 206 with positively charged Lysine[28]. Several phenomena related to protein clustering have been attributed to GFP dimerization, and use of non-dimerizing GFP mutants in those cases helped alleviate clustering and allow better analysis[28; 29; 30].

We were interested in the possibility that such effects might complicate studies that use arrays to analyze chromosome pairing. If two arrays are present within one cell, they often pair with one another as seen in yeast[19; 20; 21] and plants[22; 23]. One systematic study showed that pairing of arrays occurs regardless of their genomic location[31]. Such effects could reflect a natural underlying tendency of the corresponding loci to pair with one another and/or pairing mediated by DNA/DNA interactions of the array. However, one systematic study, using FISH instead of fluorescence to monitor array positions, suggested that such pairing can depend on the presence of repressor-GFP fusion protein[32]. We decided to study the phenomenon of array pairing further by making a mutated version of TetR-GFP with disrupted GFP dimerization, TetR-A206KGFP or “TetR-kGFP”. We report that using this mutated protein eliminates pairing of *tetO*-arrays *in vivo*. Our findings argue that pairing of arrays is caused by GFP dimerization and provide an improved tool for tagging chromosomal loci with a low pairing background.

RESULTS

We analyzed a system in which a pair of *tetO* arrays was integrated at ectopic (unrelated) positions in yeast cells (Figure 1). *tetO* array at *LYS2* locus consists of 240 copies of a DNA fragment, which contains 19 bp *tetO* binding site and a 23 bp spacer[13]. *tetO* array at *HIS4* locus consists of 120 DNA fragments, each one of which contains the 19 bp *tetO* binding site and a unique 10 bp spacer[16]. We examined the spatial juxtaposition of these arrays in the presence of either TetR-GFP fusion protein encoding wild-type GFP, which has a strong propensity to form dimers, or the same fusion protein in which the GFP portion carried a single mutation, A206K, which is known to reduce GFP/GFP dimerization[28].

We first assessed pairing of two *tetO* arrays using 3C (Chromosome Conformation Capture) analysis[33], which permits evaluation of array relationships regardless of the presence or nature of any fluorescent fusion protein (Figure 2). We compared the spatial proximities of two genomic loci in a wild type strain carrying no *tetO* arrays (case 1), and three strains in which both loci are marked with *tetO* arrays but carried either no repressor-GFP protein (case 2), non-mutated TetR-GFP protein (case 3) or mutated TetR-kGFP protein (case 4). If the non-mutated TetR-GFP protein is present, array-tagged loci exhibit robust pairing, well above the background seen in the absence of any array (compare case 3 and case 1). However, in the absence of any TetR-GFP protein or in the presence of TetR-kGFP (TetR-GFP-A206K), pairing occurred at the same level as in a strain with no arrays.

We also compared TetR-GFP and TetR-kGFP strains (cases 3 and 4 in Figure 2) by fluorescence microscopy of fixed whole cells (Figure 3). In the presence of the TetR-GFP protein, most cells exhibited a single fluorescent focus, indicating that the two arrays were usually paired. In contrast, in the presence of the TetR-kGFP mutant protein, most cells had two foci, indicating that arrays were no longer (or less frequently) paired (note that in the case of cells with 2 foci, the difference in brightness of the two foci is due to the difference in the length of the two arrays). Taken together, these results suggest that GFP/GFP dimerization can mediate stable pairing of loci located on different, non-homologous chromosomes in the yeast nucleus.

Another variant of FROS involving *tetO* arrays and TetR fusion protein is TetR-tdTomato[34], which has two units of red fluorescent protein attached to TetR, thus it can form intra-molecular dimers rather than inter-molecular dimers (Figure 1B). If fluorescent protein mediated interaction was indeed responsible for pairing of arrays, arrays marked with TetR-tdTomato should exhibit lower level of pairing than those marked with TetR-GFP because of the aforementioned feature of tdTomato. To see if this is true, we included cells with two ectopic *tetO* arrays and TetR-tdTomato in our cytomogical analysis (Figure 3). As we expected, the majority of cells with TetR-tdTomato had two foci, indicating that the two *tetO* arrays were not paired. Interestingly, the fraction of cells with 1 focus in the strain with TetR-tdTomato was slightly higher than that of the strain with TetR-kGFP, which might be due to the fact that TetR-tdTomato might have some residual ability to form inter-molecular dimers, whereas positive charge on TetR-kGFP has a repulsive effect on protein-protein interaction.

Arrays are widely used to study pairing of homologous chromosomal loci, *e.g*. during meiosis[17; 19; 21]. The above evidence, in accord with earlier studies[31; 32], suggests that GFP-mediated pairing could complicate such analysis. We thus used cytological analysis to examine array status in diploid yeast cells carrying *tetO* arrays integrated at allelic loci on homologous chromosomes in the presence of either non-mutant TetR-GFP or TetR-kGFP protein (Figure 4). Almost all cells expressing the non-mutant TetR-GFP protein exhibited one focus whereas the majority of cells with the mutated TetR-kGFP protein had two foci. Thus, GFP/GFP dimerization creates a significant background of protein-mediated pairing.

Arrays have also been used to look at the dynamics of sister chromatids in several organisms[13; 14; 15; 18]. To ask whether GFP/GFP pairing might enhance sister association, we compared diploid yeast strains carrying a single *tetO* array (on one of the homologous chromosomes), and non-mutant and mutant versions of TetR-GFP, after nocodazole-mediated arrest after replication, at G2/M stage (Figure 5). Sister loci exhibit increased separation in such cells, as compared to untreated cells, as seen by FISH[35]. Comparing cells with TetR-GFP and TetR-kGFP, we find that the fraction of two-focus cells is significantly lower in the strain carrying the non-mutant TetR-GFP, suggesting that protein-mediated pairing can oppose such separation.

DISCUSSION

Our data suggests that GFP-mediated dimerization of repressor-GFP fusion proteins can promote substantial levels of *in vivo* pairing of FROS-marked repetitive arrays, and that this background is substantially reduced by using a fusion protein carrying kGFP instead. These findings are of interest for the following four reasons.

First, we show that GFP dimerization can confer significant pairing of ectopic, allelic and sister loci, thus providing a cautionary note regarding the execution and interpretation of array-based studies of pairing phenomena. We note, however, that our findings do not compromise evidence for pre-meiotic and early meiotic pairing of homologous loci in yeast, which has been detected previously using FISH, Lox/Cre recombination and 3C analysis in the absence of arrays[33; 35; 36; 37].

Second, our kGFP system offers an improved tool for array-based analysis of intra-nuclear positions of chromosomal loci that permits use of monomeric green-fluorescent GFP. This system offers a complement to the self-dimerizing red fluorescent protein system provided by tdTomato. Given that all three proteins promote different levels of array pairing, one should exercise caution when comparing GFP, kGFP and tdTomato marked arrays. It is worth mentioning that another way to reduce pairing of arrays is by decreasing their length:

in our hands, 80-unit long arrays marked with non-mutated repressor-GFP protein had reduced level of pairing compared to 120-unit long ones, and 30-unit long ones showed no detectable pairing when integrated at ectopic loci, however in the latter case detection becomes more challenging (data not shown).

Third, protein-mediated interactions within and between chromosomes are important for many biological processes (reviewed in[7]). Pairing of arrays by way of repressor-GFP dimerization provides yet another example of such process.

Fourth, GFP/GFP dimerization could potentially be used as a tool for further dissecting such processes by the introduction of relatively weak artificial tether(s) in chromosomes. Tetramerizing LacI-GFP was used before for this purpose, but the tether created in that case was a much stronger one[14].

When the phenomenon of array pairing was first systematically analyzed, it was proposed that the basis for such pairing was DNA-DNA interaction[31]. Another study suggested that pairing can depend on the presence of repressor-GFP fusion protein, but the molecular mechanism behind the pairing was still not known[32]. Our observations confirm and extend the latter study by showing that the molecular mechanism behind array pairing is dimerization of GFP. We would like to note that our findings do not extend to all cases of chromosome pairing, which can be protein-dependent such as in the case of pairing centers[38], as well as DNA-DNA dependent, such as in the case of genome-wide dsb-independent pairing[37].

In the case of ectopic *tetO* arrays and the new mutated TetR-kGFP protein, vast majority of cells had 2 foci indicating that the two arrays were not paired. However, in the case of allelic *tetO* arrays and the new mutated TetR-kGFP protein, there was still significant fraction of cells with 1 focus. This difference could arise from residual GFP dimerization being detected in the allelic but not ectopic case perhaps due to more frequent contacts between allelic vs ectopic loci owing to genome-wide dsb-independent pairing of homologous chromosomes[37] and/or the fact that allelic loci are located at similar “latitude” in the yeast nucleus[10].

One puzzling observation is that while two *tetO* arrays pair with one another and two *lacO* arrays pair with one another, a *tetO* array and a *lacO* array don't pair[31]. How can this fact be explained if GFP dimerization is responsible for pairing of arrays? The explanation might come from the differences in the way the two repressor-GFP fusion proteins were constructed (Figure 6). TetR-GFP fusion protein was made by placing TetR on the N-terminus of GFP[13], while GFP-LacI fusion protein was made by placing LacI on the C-terminus of GFP[14]. Since two molecules of GFP can dimerize in an anti-parallel way[26; 27], one can imagine that such dimerization would be permitted in the case of having repressor molecules on the similar ends of two GFP molecules and restricted in the case of having repressor molecules on different ends of two GFP molecules because of steric clash between the two repressor molecules (Figure 6). While we tried to test this explanation by re-making TetR-GFP construct into GFP-TetR (which according to our hypothesis should allow for pairing of a *tetO* array and a *lacO* array, being topologically compatible with GFP-LacI), we were unable to produce a functional fusion protein, presumably due to compromised DNA binding.

MATERIALS AND METHODS

Construction of pTetR-kGFP plasmid

Plasmid pTetR-kGFP was constructed by PCR using plasmid pTetR-GFP, which expresses TetR-GFP fusion protein under the control of *URA3* promoter[13]. GCC codon, which codes for Alanine 206 in GFP was replaced with AAA codon for Lysine. No other changes were made. The plasmid was cut with AflII and integrated into *LEU2* locus.

Strains

All strains were diploid derivatives of *S. cerevisiae* SK1 background *ho::hisG/”*, *ura3/”*. KMY52 *HIS4::LEU2/his4X::LEU2, leu2/”* KMY198 lys2::tetO240::URA3/LYS2, HIS4::LEU2-tetO120/HIS4, leu2/” KMY179 lys2::tetO240::URA3/LYS2, HIS4::LEU2-tetO120/HIS4, LEU2:pTetR-GFP/leu2 KMY248 lys2::tetO240::URA3/LYS2, HIS4::LEU2-tetO120/HIS4, LEU2:pTetR-**k**GFP/leu2 KMY178 lys2::tetO240::URA3/LYS2, HIS4::LEU2-tetO120/HIS4, LEU2:pTetR-tdTomato/leu2 KMY172 HIS4::LEU2-tetO120/ his4X::LEU2-tetO120, LEU2:pTetR-GFP/leu2 KMY247 HIS4::LEU2-tetO120/ his4X::LEU2-tetO120, LEU2:pTetR-**k**GFP/leu2 KMY299 *lys2::tetO240::URA3/LYS2, LEU2:pTetR-GFP/leu2* KMY300 *lys2::tetO240::URA3/LYS2, LEU2:pTetR-**k**GFP/leu2*

Nocodazole arrest

4 ml YPD was inoculated with a single colony of yeast and grown overnight at 30°C, after which 25 μl of the overnight culture was transferred into 25 ml YPD and grown for 4 hours. 5 ml of culture was transferred into a fresh tube and 7.5 μl of 10 mg/ml nocodazole (Sigma M1404) solution in DMSO was added (15 μg/ml final concentration). After 2 h, G2/M arrest (large budded cells with DAPI-stained body at the neck) was confirmed, and cells were harvested and fixed for microscopy.

3C assay

3C assay was done exactly as in[39]. For each 3C template, the intensity of *signal* PCR product was normalized to the intensity of *normalization* PCR product and averaged among the triplicates. p-values for the two-sample t-test were calculated in R. *Signal* PCR primers were: LYS2-RX-I TGAAATCAGATTAGTTAGCGTTCGTAACC and KM-5f-1 TAATCGGTCGTCAGCCAACGTGAGAGTGTC; *cis* PCR primers were KM4a ACACTATCAGACCCTACAGTTAAGGAGAAA and KM9a-2 AAGCAAATGGCGTCCAAAATGTTCGACTTA.

Microscopy

Yeast was grown in YPD media to mid-logarithmic phase and fixed in 40% Ethanol / 0.1M sorbitol (for sister chromatid experiment, nocodazole-arrested cultures [above] were used). Microscopy was performed on an inverted microscope (Nikon Ti-e) with a 100x oil immersion objective. 3D z-stacks were taken at 200nm intervals for a total of 20 z-steps. 3D images were transformed to 2D by maximum intensity projection in Metamorph and spots were manually counted in ImageJ. 3 independent cultures were scored for each case; 500 cells were scored per each sample. Cells with ambiguous number of foci, as well as cells with zero foci or more than 2 foci were ignored (the fraction of such cells did not exceed 5% in all samples). p-values for the two-sample t-test were calculated in R.

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Highlights

* Chromosomally-integrated arrays of repetitive sequences pair with each other
* TetR-kGFP (TetR-A206KGFP) abolishes pairing of *tetO*-arrays *in vivo*
* Pairing of arrays is caused by protein-protein interactions

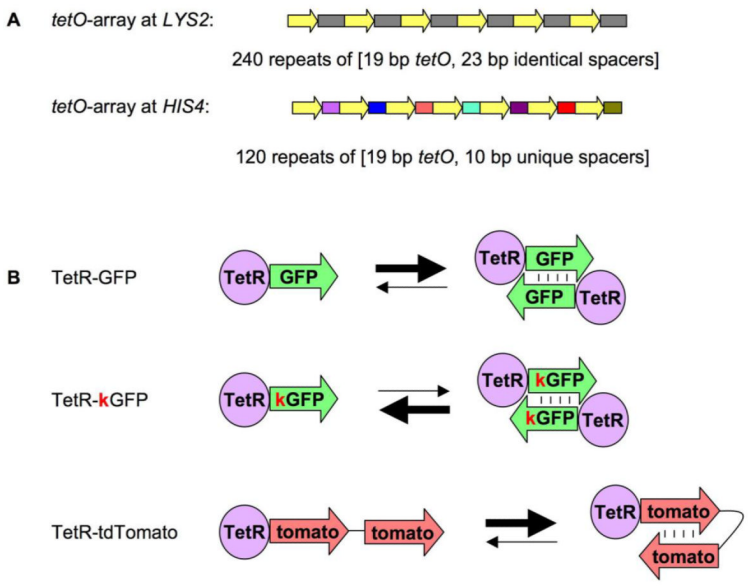

Figure 1. Arrays and proteins used in this study

A. Arrays used in this study.

The two arrays used in this study are: *tetO* array at *LYS2* locus and *tetO* array at *HIS4* locus. The former consists of 240 copies of a DNA fragment, which contains 19 bp *tetO* binding site and a 23 bp spacer (thus all spacers in the array are identical). The latter consists of 120 DNA fragments, each one of which contains the 19 bp *tetO* binding site and a unique 10 bp spacer (thus all spacers in the array are different).

B. Proteins used in this study.

The three proteins used in this study are: TetR-GFP, TetR-kGFP and TetR-tdTomato. TetR-GFP has a strong tendency to form inter-molecular dimers due to GFP dimerization. TetR-kGFP carries A206K mutation, which disrupts this property. TetR-tdTomato has two units of Tomato attached to TetR, thus it can form intra-molecular dimers instead of inter-molecular dimers.

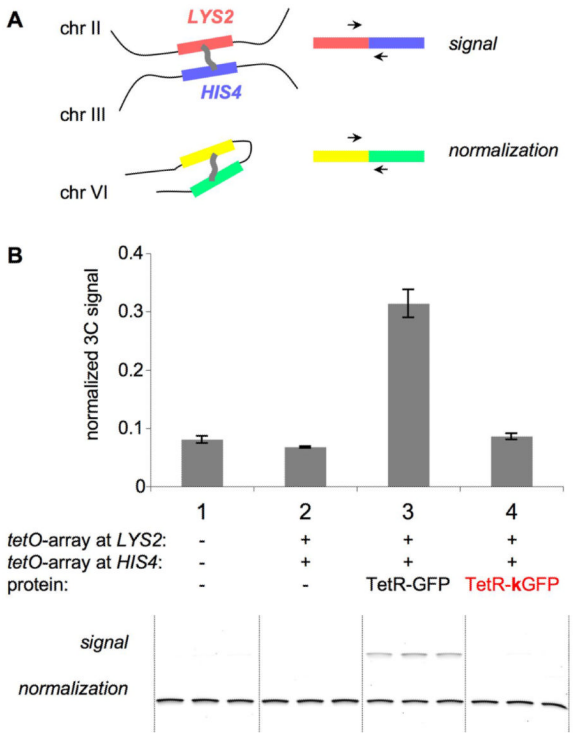

Figure 2. 3C assay of spatial proximity between chromosomal loci

A. Schematic representation of the 3C assay.

3C assay relies on chromatin crosslinking and subsequent PCR analysis of ligation junctions between crosslinked segments. *Signal* PCR assays spatial proximity of *LYS2* locus on chromosome II and *HIS4* locus on chromosome III, where *tetO* arrays are integrated. *Normalization* PCR, which assays spatial proximity between two randomly chosen chromosomal segments located in *cis*, is used to control for the efficiency of the procedure in all samples.

B. 3C assay of spatial proximity between *HIS4* and *LYS2* loci.

Below – gel (reactions are done in triplicates, such that 3 PCR reactions were set up using 1 3C template), above – quantification of gel products. Case 1 – control WT strain without arrays and repressor proteins. Cases 2, 3, 4 – *tetO* arrays are present at *HIS4* and *LYS2* loci. Case 2 – no repressor protein, case 3 – TetR-GFP, case 4 – TetR-kGFP.

$p(1,2) = 8.64 \times 10^{-2}$; $\underline{p(1,3) = 3.44 \times 10^{-3}}$; $p(1,4) = 4.09 \times 10^{-1}$; $\underline{p(2,3) = 4.60 \times 10^{-3}}$; $p(2,4) = 3.4 \times 10^{-2}$; $\underline{p(3,4) = 3.92 \times 10^{-3}}$ (all p-values that include sample 3 are < 0.01; underlined).

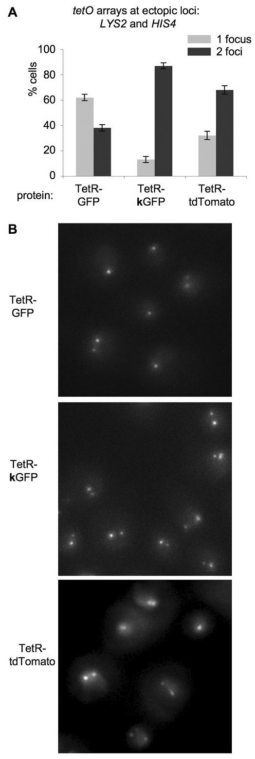

Figure 3. Microscopic analysis of cells with two ectopic *tetO* arrays and TetR-GFP, TetR-kGFP or TetR-tdTomato protein

A. Quantitation of the microscopic analysis.

Percentages of cells with 1 focus and 2 foci are plotted for three strains, all of which have two *tetO* arrays located at *HIS4* and *LYS2* loci, plus one of the proteins: TetR-GFP, TetR-kGFP or TetR-tdTomato. Light grey bars – percentage of cells with 1 focus, dark grey bars – percentage of cells with 2 foci. $p(1,2) = 3.45\times10^{-5}$; $p(1,3) = 7.3\times10^{-4}$; $p(2,3) = 3.38\times10^{-3}$ (all p-values are < 0.01).

B. Representative images of cells.

In the case of cells with 2 foci, the difference in brightness of the two foci is due to the difference in the length of the two arrays (Figure 1A).

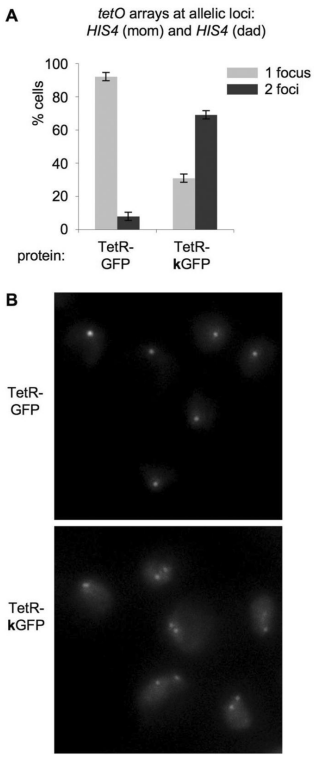

Figure 4. Microscopic analysis of cells with two allelic *tetO* arrays and TetR-GFP or TetR-kGFP protein

A. Quantitation of the microscopic analysis.

Percentages of cells with 1 focus and 2 foci are plotted for two strains, both of which have two *tetO* arrays located at *HIS4* loci on two homologous chromosomes, plus one of the proteins: TetR-GFP or TetR-kGFP. Light grey bars – percentage of cells with 1 focus, dark grey bars – percentage of cells with 2 foci. $p=1.64 \times 10^{-5} < 0.01$.

B. Representative images of cells.

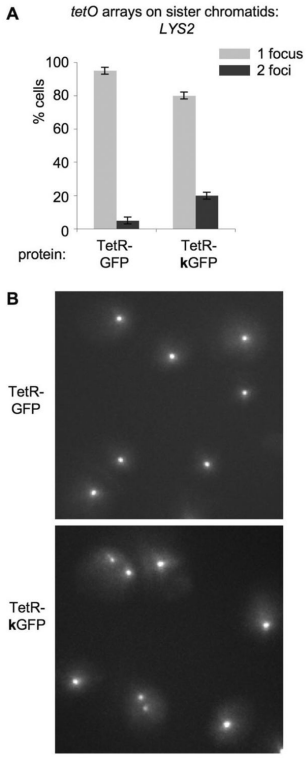

Figure 5. Microscopic analysis of G2/M-arrested cells with *tetO* array at *LYS2* locus and TetR-GFP or TetR-kGFP protein

A. Quantitation of the microscopic analysis.

Percentages of cells with 1 focus and 2 foci are plotted for two strains, both of which have *tetO* array located at *LYS2* locus, plus one of the proteins: TetR-GFP or TetR-kGFP. Cells were arrested in G2/M with nocodazole, so each cells contains two sister chromatids, each of which has *tetO* array at *LYS2*. Light grey bars – percentage of cells with 1 focus, dark grey bars – percentage of cells with 2 foci. $p=2.22 \times 10^{-3} < 0.01$.

B. Representative images of cells.

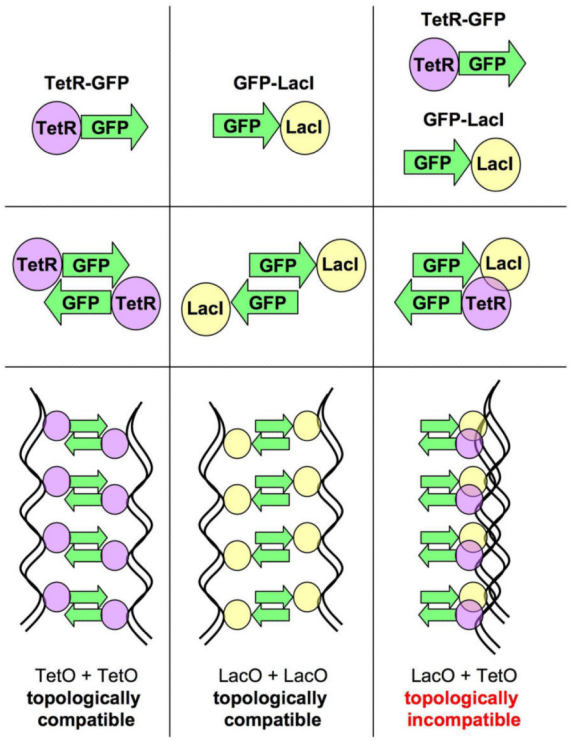

Figure 6. Model

Two GFP molecules are capable of forming anti-parallel dimers. Two molecules of TetR-GFP can easily dimerize with each other, thus pairing two *tetO* arrays. Likewise, two molecules of GFP-LacI can easily dimerize with each other, thus pairing two *lacO* arrays. However, a TetR-GFP molecule cannot easily dimerize with a GFP-LacI molecule because of steric clash between TetR and LacI, which occurs because TetR and LacI will be on the same side of the dimer (as opposed to TetR-GFP/TetR-GFP or GFP-Lac/GFP-LacI dimers, in which two repressor molecules will be on different sides of the dimer).