

# Preferential synapsis of *loxP* sites drives ordered strand exchange in Cre-*loxP* site-specific recombination

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The bacteriophage P1 Cre recombinase catalyzes site-specific recombination between 34-base-pair *loxP* sequences in a variety of topological contexts. This reaction is widely used to manipulate DNA molecules in applications ranging from benchtop cloning to genome modifications in transgenic animals. Despite the simple, highly symmetric nature of the Cre-*loxP* system, there is strong evidence that the reaction is asymmetric; the 'bottom' strands in the recombining *loxP* sites are preferentially exchanged before the 'top' strands. Here, we address the mechanistic basis for ordered strand exchange in the Cre-*loxP* recombination pathway. Using suicide substrates containing 5'-bridging phosphorothioate linkages at both cleavage sites, fluorescence resonance energy transfer between synapsed *loxP* sites and a Cre mutant that can cleave the bridging phosphorothioate linkage but not a normal phosphodiester linkage, we showed that preferential formation of a specific synaptic complex between *loxP* sites imposes ordered strand exchange during recombination and that synapsis stimulates cleavage of *loxP* sites.

The bacteriophage P1 Cre recombinase catalyzes site-specific recombination between 34-base-pair (bp) *loxP* sites<sup>1</sup>. This reaction is used by P1 to maintain stable inheritance of the episomal genome in the lysogenic state<sup>2</sup>. Cre is a member of the tyrosine recombinase family of proteins found in bacteria and yeast, whose functions include integration and excision of viral genomes from bacterial host chromosomes, resolution of multimeric plasmids and chromosomes generated by homologous recombination, amplification of plasmid copy number, regulation of gene expression and transposition<sup>3,4</sup>. This family of enzymes shares a common mechanism of stepwise strand exchange during recombination, where one pair of DNA strands is first exchanged between recombining sites to form a four-way Holliday junction (HJ) intermediate, and the second pair of strands is then exchanged during resolution of the HJ intermediate to form recombinant products (Fig. 1a)<sup>4,5</sup>.

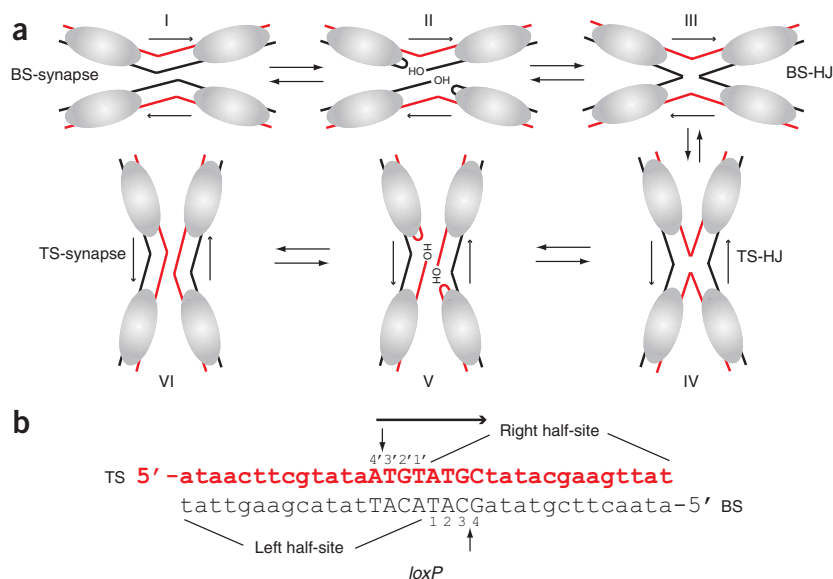
Cre is generally viewed as the simplest of the well-studied tyrosine recombinases. Efficient recombination does not require a specific DNA substrate topology, and the *in vitro* reaction does not require protein accessory factors<sup>6</sup>. In contrast, the more complex tyrosine recombinases such as the integrase protein from bacteriophage  $\lambda$  and the bacterial XerC and XerD recombinases require additional proteins to carry out recombination<sup>5,7,8</sup>, and strands are exchanged between recombining sites in a strictly defined order<sup>9-11</sup>. Both the minimal nature of the Cre-*loxP* system and its ability to function effectively in eukaryotic cells has led to its widespread use as a tool to rearrange genetic elements in a broad range of organisms and to manipulate DNA molecules *in vitro*<sup>12</sup>. Cre has also served as a useful model system for structural and biochemical analyses of the protein-DNA intermediates formed in the tyrosine recombinase site-specific recombination pathway<sup>13,14</sup>.

Given the simplicity of the Cre-*loxP* system, it might be expected that there would be no preference for which strand is first exchanged between recombining *loxP* sites and that Cre would be able to efficiently recombine substrates in either the forward or reverse direction as shown (Fig. 1a). The only known source of asymmetry in this system is the 8-bp segment at the center of *loxP*, which is flanked by recombinase binding elements arranged as perfect inverted repeats (Fig. 1b). However, data from several laboratories indicate that Cre does exchange *loxP* strands in a specific order, although there are conflicting conclusions regarding which direction is preferred<sup>15-19</sup>. The strongest evidence is from studies with wild-type Cre and unmodified *loxP* sites, which indicate that the *loxP* bottom strands are exchanged first in the reaction<sup>16,19</sup>.

We addressed the issue of ordered strand exchange in the Cre-*loxP* system in the context of the synaptic complex that forms at the start of the recombination pathway. Using suicide *loxP* sites modified to contain 5'-bridging phosphorothioate linkages at both cleavage sites, we showed that Cre preferentially cleaves the bottom strands of *loxP* and that this preference is dependent on synapsis of the sites. In contrast, top-strand (TS) cleavage of *loxP* sites is largely independent of synapsis. Fluorescence resonance energy transfer (FRET) between synapsed *loxP* sites that have been labeled with donor and acceptor fluorophores indicated that the synapse committed to bottom-strand exchange (the BS synapse) is preferred over the synapse committed to TS exchange (the TS synapse) in solution, providing an explanation for the observed synapsis-dependent cleavage bias. The use of bridging phosphorothioate substrates with the normally inactive Cre K201A mutant has provided valuable insights into the asymmetric nature of the Cre-*loxP* reaction and indicates a role as general acid catalyst for Lys201 in the Cre active site. Finally, we showed that substitutions that

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**Figure 1** Cre-*loxP* site-specific recombination. **(a)** Schematic of the recombination pathway. Red lines represent the top strands and black lines the bottom strands as defined in **b**. Arrows above or below the *loxP* sites indicate the direction of the site, as defined in **b**. TS and BS synapse refer to synaptic complexes where the top or bottom strands are in a cleaving configuration, respectively. Intermediates I–VI and details of the cartoon are discussed in the text. **(b)** The 34-bp *loxP* site, with inverted repeat recombinase binding elements in lowercase and the 8-bp spacer in uppercase. Vertical arrows indicate the cleavage positions and the horizontal arrow defines the site direction as used in **a** and in other figures. Numbering and TS/BS definitions follow those previously described<sup>42</sup>, where primed numbers are used here in place of negative numbers.

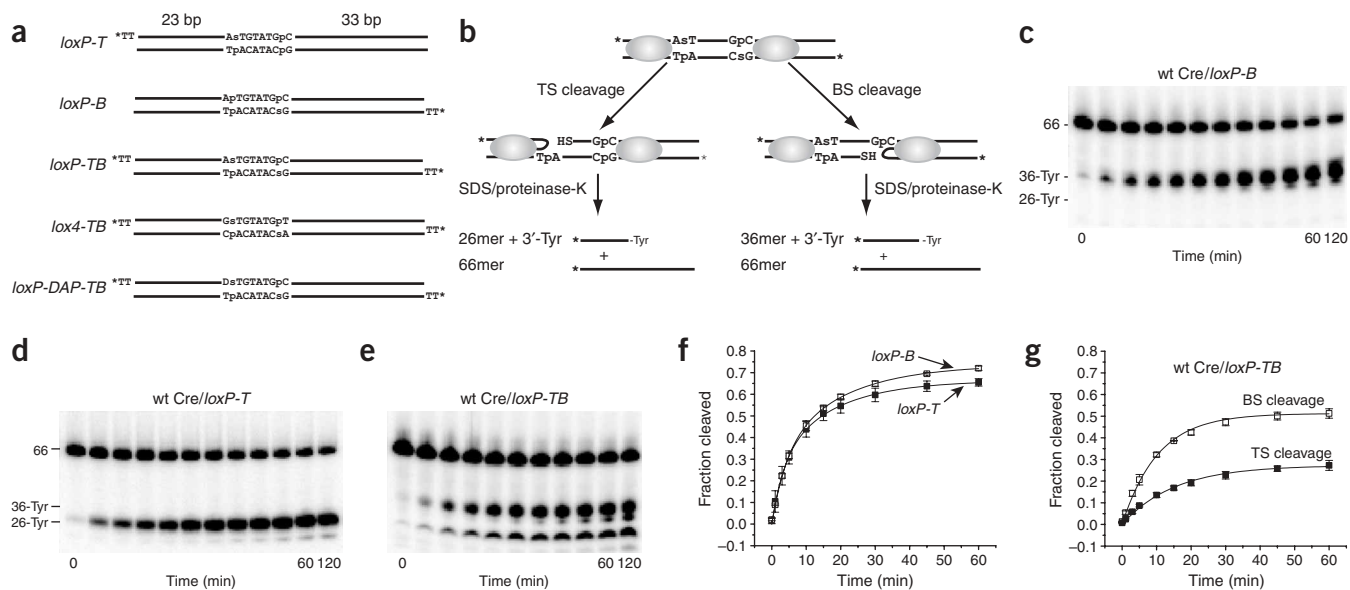
symmetrize or swap the scissile bases lead to sites that are less efficiently recombined compared to wild-type *loxP*, indicating that an ordered strand-exchange pathway has an important functional role in Cre-*loxP* recombination.

## RESULTS

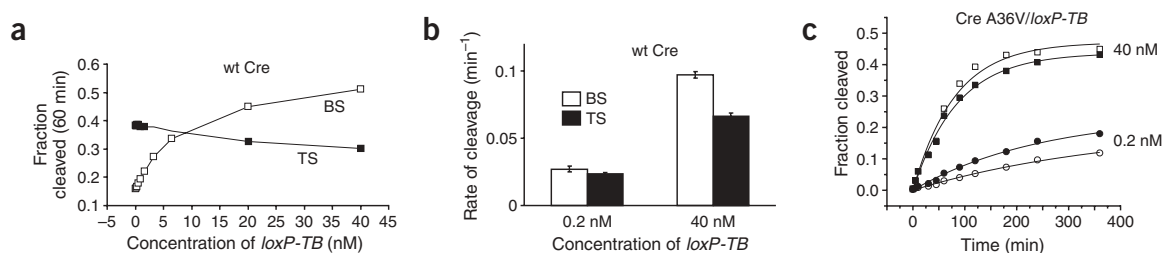
### Cre initiates recombination by cleaving the *loxP* bottom strands

To directly address the question of which strand is preferentially cleaved to initiate Cre-*loxP* site-specific recombination, we prepared DNA substrates containing 5'-bridging phosphorothioate linkages at one or

eukaryotic type Ib topoisomerases has been well established<sup>20–23</sup>. Both  $\lambda$ -integrase and vaccinia virus topoisomerase will readily cleave substrates containing the 5'-thio linkage, but the rate of re-ligation or strand-exchange ligation is sufficiently slow that the cleavage reaction is effectively irreversible<sup>21</sup>. Recombination substrates containing this modification at a specific cleavage site are therefore trapped after cleavage at that site as covalent 3'-phosphotyrosine intermediates (for example, intermediate II in Fig. 1a) that are unable to efficiently re-ligate to re-form the original duplex substrates or to undergo strand-exchange ligation to form the HJ intermediate.



**Figure 2** Cleavage of *loxP* suicide substrates. **(a)** The *loxP* substrates used in cleavage assays. The 's' linkages indicate 5'-bridging phosphorothioates; 'p' linkages are normal phosphodiester, and 'D' represents 2,6-diaminopurine. <sup>32</sup>P 5'-end labels are indicated by asterisks. Full DNA sequences are shown in **Supplementary Figure 1**. **(b)** Schematic of the cleavage assay and the ssDNA products expected in denaturing PAGE analysis. **(c–e)** Denaturing PAGE following cleavage assays with wt Cre and the *loxP-B*, *loxP-T* and *loxP-TB* substrates, respectively. **(f)** Data from **c** and **d** plotted together; curves are two-exponential fits and do not represent a specific model. **(g)** TS and BS cleavage data from **e**; curves are pseudo-first-order kinetics fits of the form  $y = f_{\max}(1 - e^{-kt})$ , where  $f_{\max}$  is the 2-h end point (data not shown). The rate constants for formation of BS- and TS-cleavage products in **g** are  $0.0969 \pm 0.002 \text{ min}^{-1}$  and  $0.0661 \pm 0.003 \text{ min}^{-1}$ , respectively. Error bars in **f** and **g** and in **Figures 3–7** represent two standard deviations based on 3–5 independent experiments. wt Cre, wild-type Cre.



**Figure 3** Effects of synapsis on *loxP* cleavage. **(a)** Cleavage of *loxP-TB* sites as a function of *loxP* concentration. The 60-min time points from reaction time courses performed as in **Figure 2e** are shown. Concentrations tested were 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 20 and 40 nM. **(b)** Rates of *loxP-TB* cleavage by wt Cre at 0.2 nM and 40 nM substrate concentration. **(c)** Longer time course of *loxP-TB* cleavage by Cre A36V at 0.2 nM (circles) and 40 nM (squares) substrate concentration. Open symbols represent BS cleavage and filled symbols represent TS cleavage. Reaction time courses were fit with a single exponential equation as in **Figure 2g**, where  $t_{\max}$  is the 6-h end point. wt Cre, wild-type Cre.

We prepared *loxP* substrates for Cre recombinase in which the TS ApT and/or the BS GpC linkages have been modified to contain a 5'-bridging phosphorothioate using a chemical ligation approach<sup>24,25</sup>. Details of the oligonucleotide syntheses are provided as **Supplementary Methods** online. The sequences flanking the *loxP* site in these substrates are of different lengths, allowing us to readily distinguish and quantify TS versus BS cleavage by denaturing electrophoresis following proteinase-K digestion of the covalently bound Cre-DNA intermediates (**Fig. 2a,b**). Cleavage assays were performed at 40 nM DNA substrate concentration and saturating concentrations of Cre. Because the affinity of Cre for the *loxP* sequence is quite high<sup>6,26</sup>, *loxP* sites in the assays described here are all bound by two Cre subunits. We have measured the synapsis dissociation constant for wild-type Cre and *loxP* sites to be  $\sim 10$  nM using both sedimentation equilibrium ultracentrifugation (data not shown) and electrophoresis methods (an example is shown in **Supplementary Fig. 2** online), and this value agrees well with an estimate from fitting a kinetic model to intramolecular Cre-*loxP* recombination<sup>26</sup>. Thus, more than half of the *loxP* sites are expected to exist as synaptic complexes at any given time in this assay, as the  $\text{Cre}_2\text{loxP}$  concentration is greater than  $K_d$ .

The results of a cleavage assay with wild-type Cre and *loxP* substrates containing the bridging sulfur modification in the top strand (*loxP-T*), bottom strand (*loxP-B*) or both strands (*loxP-TB*) of *loxP* are shown in **Figure 2**. Both TS- and BS-modified sites are efficiently cleaved to form covalent intermediates (**Fig. 2c,d**), and the cleavage kinetics are similar (**Fig. 2f**). For the *loxP-T* and *loxP-B* substrates, this assay does not provide an indication of which strand is cleaved first during recombination, because cleavage of the 5'-phosphorothioate linkage could occur during the first strand exchange to make the HJ intermediate or during the second strand exchange to resolve the HJ intermediate. In control reactions with the inactive Cre mutant Y324F, we observed no cleavage products after 2-h incubations (data not shown).

When both cleavage sites were modified with the 5'-bridging sulfur substitution (*loxP-TB*), Cre showed a  $\sim$ two-fold preference in the extent of BS cleavage at the GpC step of *loxP* (**Fig. 2e,g**). This result is in sharp contrast to the cleavage preference observed with wild-type Cre on nicked suicide substrates at high concentrations, where TS cleavage is more efficient<sup>17</sup>. Both TS and BS cleavage follow pseudo-first-order kinetics (**Fig. 2g**), which implies that association of Cre-bound *loxP* sites to form synaptic complexes is either fast relative to cleavage of the sites or that association of the sites is not required for cleavage.

### BS cleavage preference is synapsis dependent

The most naive interpretation of the modest two-fold preference for BS cleavage observed in the above experiments is that Cre initiates recombination 66% of the time by BS cleavage and the remainder by TS cleavage. However, it is currently unknown how efficiently Cre will cleave an isolated *loxP* site in the absence of synapsis. With normal substrates, topoisomerase-like nicking of isolated *loxP* sites by Cre is expected to be efficiently repaired by the reverse ligation reaction, and the absence of substantial topoisomerase activity by Cre on normal sites<sup>6,27</sup> suggests that if cleavage does occur, re-ligation is fast relative to strand rotation. If isolated *loxP* site cleavage occurs at a rate that is comparable to cleavage within an assembled recombination complex, then some of the irreversible TS- or BS-cleavage activity indicated in **Figure 2g** may not be relevant to the recombination pathway.

We therefore wanted to determine whether strand-cleavage preference and the rate of cleavage depend on synapsis of the *loxP* sites. A synapsis-dependent or synapsis-stimulated cleavage mechanism would imply that cleavage of chromosomal *loxP* sites is suppressed in the absence of a recombining partner and activated upon formation of a functional recombination assembly. To determine if the strand-cleavage preference is affected by synapsis, we compared the extent of cleavage by wild-type Cre after 60 min at concentrations of *loxP-TB* ranging from 100 pM to 40 nM (**Fig. 3a**). Remarkably, the fraction of BS-cleavage products increases sharply as a function of *loxP* concentration, but the fraction of TS-cleavage product formed is relatively independent of *loxP* concentration below the synapsis  $K_d$  ( $\sim 10$  nM) and then decreases slightly as BS-cleavage products begin to predominate. Extrapolation to zero concentration indicates that in the absence of synapsis, TS cleavage occurs exclusively, with BS cleavage virtually nonexistent. Aliquots of the reaction mixtures analyzed to give the data shown in **Figure 3a** were also run on non-denaturing polyacrylamide gels to verify the expected increase in synaptic complex formation as the concentration of *loxP* was increased (**Supplementary Fig. 2** online).

To determine if synapsis affects the rate of strand cleavage, we compared time courses for reactions at 200 pM and 40 nM *loxP-TB* substrate concentrations (**Fig. 3b**). The pseudo-first-order rate constant for BS cleavage at 200 pM substrate ( $0.022 \pm 0.005 \text{ min}^{-1}$ ) increases by roughly five-fold at the higher *loxP* concentration ( $0.097 \pm 0.002 \text{ min}^{-1}$ ), suggesting that synapsis does in fact stimulate cleavage of the sites. The rate of TS cleavage also increases, by roughly three-fold. In the case of TS cleavage, it is likely that we are comparing the rate of primarily isolated *loxP* cleavage (at 200 pM) to a composite rate of isolated and synapsed *loxP* cleavage (at 40 nM). Together, the data suggest that TS cleavage occurs in both the presence and the

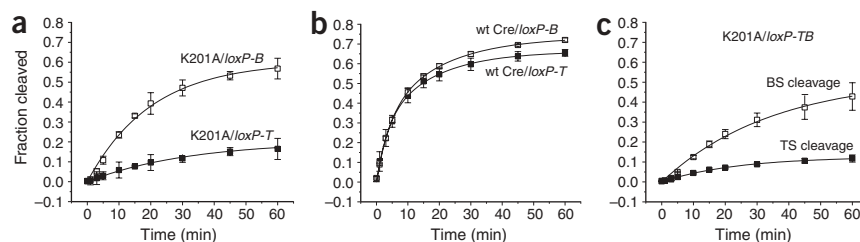
absence of synapsis, whereas BS cleavage occurs only in the context of a synaptic complex. Interestingly, the extent of formation of TS- versus BS-cleavage products at the different concentrations does not correlate in a simple way with their rates of formation. Presumably, this is a reflection of competition between three distinct reactions, with different stabilities of the respective species involved.

We also analyzed the cleavage activity of a Cre mutant (A36V) known to be partially defective in synapsis of *loxP* sites but catalytically competent to cleave HJ intermediates<sup>15,28</sup>. The A36V mutant binds with wild-type affinity to *loxP* sites but is inefficient in carrying out a complete recombination reaction. Under the same conditions used to assay wild-type Cre, the A36V mutant shows a reduced ability to cleave *loxP* sites, with only a small preference for BS cleavage at the higher *loxP* concentration. Because of the reduced activity of this mutant, it was necessary to extend reactions to much longer time courses (Fig. 3c). The observation that TS cleavage is strongly impaired at low *loxP* concentration indicates that in addition to being defective in synapsis, the A36V mutant is also defective at cleaving an isolated *loxP* site. This result is not surprising, as the recombinase residues involved in stabilizing the synaptic complex (which include Ala36) are also likely to form protein-protein interactions on an isolated *loxP* site. These residues are expected to be involved in DNA bending and formation of a unique active site that catalyzes cleavage of either the top or bottom strand.

### Cleavage of suicide substrates with Cre K201A

The tyrosine recombinases are closely related to the eukaryotic type Ib topoisomerases, which catalyze cleavage and re-ligation of DNA strands using similar catalytic machinery, and the *Vaccinia* virus topoisomerase has served as an important model system for understanding catalysis in this superfamily<sup>29</sup>. In the *Vaccinia* virus enzyme, Lys167 has been shown to participate in general acid catalysis, providing the proton that facilitates expulsion of the 5'-bridging oxygen leaving group during DNA strand cleavage. The most compelling evidence for this role came from the strong thio-effect observed on a 5'-bridging phosphorothioate DNA substrate using a K167A mutant<sup>20</sup>. In that case, the K167A enzyme cleaved the bridging-thio substrate nearly as well as the wild-type topoisomerase, whereas the same mutant cleaves normal substrates with a rate that is  $\sim 10^4$  times slower than the wild-type enzyme. Facile cleavage of the thio-substrate by the K167A mutant was attributed to the greatly reduced pKa value of the 5'-thiol relative to the 5'-hydroxyl leaving group<sup>20</sup>. The corresponding residue in the Cre active site is Lys201. The Cre K201A mutant is inactive in cleavage and recombination of *loxP* sites but is able to synapse *loxP* sites nearly as well as wild-type Cre (data not shown).

As shown in Figure 4, the Cre K201A mutant efficiently cleaves the 5'-bridging phosphorothioate-modified *loxP-T*, *loxP-B* and *loxP-TB* substrates. These reactions were performed under identical conditions to those shown in Figure 2 for wild-type Cre using the same substrates. Cleavage of the *loxP-TB* substrate by wild-type Cre and Cre K201A show a similar bias for cleaving the bottom strands, but the pseudo-first-order rate constants for the K201A mutant are decreased somewhat compared to that observed for wild-type Cre. We therefore conclude that, like Lys167 in the *Vaccinia* virus topoisomerase, Lys201 has a role as general acid catalyst in Cre recombinase.



**Figure 4** Cleavage of 5'-bridging phosphorothioate substrates by Cre K201A. (a) Cleavage of *loxP-T* and *loxP-B* by Cre K201A. (b) Cleavage of *loxP-T* and *loxP-B* by wt Cre. (c) Cleavage of *loxP-TB* by Cre K201A, with TS- and BS-cleavage products plotted together. Curves in c are pseudo-first-order fits, as in Figure 2g, with rate constants  $0.029 \pm 0.002$  and  $0.042 \pm 0.009$   $\text{min}^{-1}$  for BS cleavage and TS cleavage, respectively. wt Cre, wild-type Cre.

In contrast to the results with the doubly modified *loxP-TB* substrate, the cleavage rate profiles for Cre K201A with the singly modified *loxP-T* and *loxP-B* substrates (Fig. 4a) were found to be quite different from those observed with wild-type Cre (Fig. 4b). For wild-type Cre, the rates and extents of cleavage for TS- and BS-substituted *loxP* sites are similar. In those cases, Cre either cleaves the sulfur-containing strands first to generate products, or the unmodified strands are cleaved and exchanged first to form the HJ intermediate and the modified strands are then cleaved to generate products. When the K201A mutant is used, product accumulates faster and to a greater extent for the *loxP-B* substrate. A reasonable explanation for this observation is that a synaptic complex corresponding to a BS cleavage configuration is favored, leading to efficient cleavage of the *loxP-B* substrate to form products. The K201A mutant is unable to cleave the bottom strands of *loxP-T*, so TS-cleavage products must arise from formation of a presumably less favored TS synapse or from cleavage of unsynapsed sites at a lower rate.

An analysis of the concentration dependence of cleavage by the K201A mutant similar to that shown in Figure 3a indicates that, like wild-type Cre, BS cleavage is strongly concentration dependent (data not shown). However, we also observed a concentration dependence for TS cleavage by K201A, suggesting that both TS and BS cleavage by this mutant may require synapsis. It is possible that Lys201 (which contacts the scissile bases through the minor groove) has a role in bending and/or organizing the unsynapsed Cre-*loxP* complex into a conformation suitable for cleavage and that the K201A mutant is unable to achieve the required configuration.

### Cre forms a preferred synaptic complex between *loxP* sites

The cleavage activity of wild-type Cre and Cre K201A on suicide substrates both strongly suggest that a synaptic complex of *loxP* sites poised for BS cleavage is preferred over a complex poised for TS cleavage and that this synaptic preference is the basis for the observed cleavage preference. To obtain independent evidence for a preferred antiparallel synaptic complex formed between Cre-bound *loxP* sites, we designed a FRET experiment that could distinguish between the TS and BS synapses. The FRET experiment is shown schematically in Figure 5a. Cleavage-deficient Cre K201A is bound to *loxP* sites carrying donor and acceptor fluorophores that are designed to be close to one another in one synaptic complex, but farther apart in the alternative complex. The fluorophore label positions in the sites were chosen so that the donor projects out of the major groove of one *loxP*-containing duplex, across the synaptic interface, toward a complementary acceptor fluorophore on the synapsed *loxP* site. Modeling of this experiment based on Cre-*loxP* synaptic complex crystal

structures<sup>18,30</sup> guided our choice of label positions on the DNA duplex in the flanking regions of the *loxP* sites. The strategy of coupling fluorophores to thymidines in the major grooves of the sites (as opposed to labeling on the 3'- or 5'-ends) also allowed us to include 10-bp sequences flanking the *loxP* site and thereby improve the stability of the DNA duplexes.

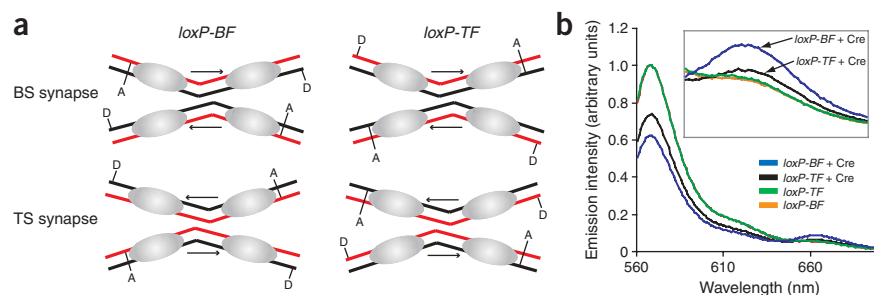
Two different dual-labeled *loxP* sites were prepared (Fig. 5a). *LoxP*-BF will have shorter distances between donor and acceptor fluorophores in a BS synapse but longer distances between fluorophores in a TS synapse. *LoxP*-TF has identical donor and acceptor fluorophore positions but in the opposite sense relative to the central 8 bp, so that the shortest donor-acceptor distances will be present in the TS synapse. Because the fluorophores are located on equivalent positions in the substrates being compared, differences in donor-acceptor distances should arise solely from the relative abundance of synapse types (TS versus BS) that are formed. The crystal structures of three independent Cre-*loxP* synaptic complexes are superimposable outside of the central 8 bp of the *loxP* site<sup>18,30</sup>, indicating that there should be no intrinsic difference between the TS and BS synapses arising from flanking DNA geometry in this experiment.

The results of the FRET experiment are shown in Figure 5b. As expected, the *loxP*-TF and *loxP*-BF substrates alone have identical fluorescence properties. Upon addition of Cre K201A to the dual-labeled *loxP* sites under conditions favoring synapsis (100 nM *loxP* site), the donor fluorescence of the *loxP*-BF probe is quenched more strongly than that of the *loxP*-TF probe. In addition, the emission fluorescence from the acceptor fluorophore is greater for *loxP*-BF than for *loxP*-TF (Fig. 5b, inset). Both observations indicate that closer donor-acceptor distances are present in the synapse formed between *loxP*-BF sites, leading to more efficient energy transfer (see Fig. 5 legend).

When this experiment was repeated with the synapsis-defective Cre A36V mutant, only a small amount of donor quenching was observed relative to DNA substrates labeled with only the donor, and the spectra were nearly identical for the Cre-bound *loxP*-TF and *loxP*-BF substrates (data not shown). No measurable acceptor emission fluorescence was observed for either substrate. These results are consistent with the data shown in Figure 3c, which indicates that synapsis is weak with little discrimination between TS and BS cleavage. Overall, the FRET experiments indicate that the Cre-*loxP* BS synapse is preferred over the TS synapse, providing independent support for a model in which ordered strand exchange is established by preferred formation of a BS synaptic complex.

### Cleavage preferences are dictated by the *loxP* sequence

Having found that Cre preferentially forms a BS-cleavage synaptic complex and initiates cleavage on the bottom strands of the *loxP* site, we asked whether specific amino acids in the recombinase were responsible for 'selecting' the BS synapse. There are two direct contacts between Cre and the bases in the 8-bp central region of the *loxP* site, based on crystal structures of the synaptic complex<sup>18,30</sup>, the covalent Cre-DNA intermediate<sup>31</sup> and the Cre-HJ intermediate<sup>17,32,33</sup>. Lys86 interacts with the scissile bases Ade4' and Gua4 through the major



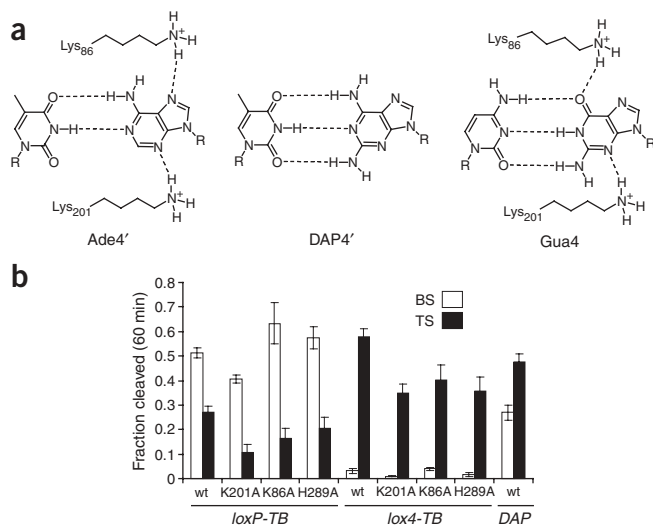
**Figure 5** Synapsis of *loxP* sites monitored by FRET. (a) Cartoon representation of the antiparallel synaptic complexes expected from *loxP*-BF and *loxP*-TF substrates. The TS and BS synapses differ by the bend direction of the sites and by the strands that are activated for cleavage in the presence of Cre. The *loxP*-BF substrate will result in more efficient energy transfer if a BS synapse is favored and the *loxP*-TF substrate will result in more efficient energy transfer if a TS synapse is favored. D = Alexafluor-546, A = Alexafluor-647, with a nominal Förster radius of  $\sim 74$  Å. Fluorophores were coupled to the C5 positions of specific Thy residues through amino linkers. DNA sequences are given in Supplementary Figure 1. (b) Fluorescence emission spectra for *loxP*-TF and *loxP*-BF sites with and without Cre K201A added, using 555 nm excitation. The inset spectrum highlights the acceptor emission at  $\sim 660$  nm. Energy transfer efficiencies are 0.23 for Cre + *loxP*-BF and 0.11 for Cre + *loxP*-TF.

groove (*loxP* numbering is shown in Fig. 1b), and Lys201 interacts with the same bases, but through the minor groove. The direct interactions involving Lys86, Lys201 and the scissile bases are shown schematically in Figure 6a.

Using the *loxP*-TB substrate, we analyzed the time course of cleavage for the K86A and K201A Cre mutants. In both cases, the same preference for cleavage of the bottom strand of the *loxP* site was observed (Fig. 6b), indicating that Cre does not select the bottom strand for initial cleavage through direct interaction with the asymmetric region of the *loxP* site. An analysis of HJ intermediates generated by Cre-*loxP* recombination had previously revealed that both wild-type Cre and the K86A mutant generated intermediates predominantly from BS exchange<sup>16</sup>. However, it was not possible to rule out a role for Lys201 in mediating this preference because Lys201 is required for strand exchange of unmodified *loxP* substrates.

We also tested the Ala mutant of His289, a conserved active site residue that may participate in general base catalysis during the phosphoryl transfer reaction<sup>13,34</sup>, although this role has not yet been rigorously investigated. We were interested in this Cre mutant because one of the two reports indicating that Cre exchanges the top strands first during recombination made use of Cre H289A to accumulate HJ intermediates, which were then analyzed to determine which strands had been exchanged<sup>17</sup>. In principle, the use of a particular Cre mutant could alter the normal order of strand exchange<sup>16</sup>. However, Cre H289A also showed a preference for BS cleavage of the *loxP*-TB substrate (Fig. 6b). The cleavage rate for H289A on the bridging phosphorothioate substrate is reduced relative to wild-type Cre, as previously reported for unmodified *loxP* sites<sup>17</sup>. The concentration dependence of cleavage by H289A is similar to that observed for wild-type Cre in Figure 3a. BS cleavage disappears at low concentration of *loxP*, whereas TS cleavage shows very little concentration dependence (data not shown).

We next asked whether the observed BS-cleavage preference could be reversed by modifying the asymmetric sequence present in the central 8 bp of *loxP*. We began by analyzing cleavage of the suicide *lox4*-TB substrate (Fig. 2a). The *lox4* sequence is identical to *loxP*, except that the Ade4' and Gua4 bases at the cleavage positions are swapped between half-sites<sup>35</sup>. When we performed assays on *lox4*-TB



**Figure 6** Determinants of cleavage preference. **(a)** Schematic of Lys86 and Lys201 interactions with the scissile Ade4' and Gua4 bases. The 2,6-diaminopurine (DAP) substituted Ade4'-Thy4' bp is shown for comparison. **(b)** Results of cleavage assays performed with Cre mutants and *loxP* variants. The 60-min time points from reaction time courses performed as in **Figure 2e** are shown. DAP refers to the *loxP*-DAP-TB substrate (**Fig. 2a**). wt, wild type.

Indeed, Cre cleaves the *loxP*-TB variant with Ade4' replaced by DAP to give products with not only a reduced bias, but a reversed bias, with TS cleavage now favored by a nearly 2:1 ratio (**Fig. 6b**).

### Scissile bases are important for efficient recombination

The bases in the 4 and 4' positions of *loxP* are not exchanged between recombining sites and therefore are not subject to testing for crossover sequence identity during recombination. In principle, substitutions in these positions should all be viable for recombination, as Lys86 would be expected to make a productive interaction with either Thy or Cyt bases and the binding affinity to the *loxP* site should not be significantly affected. Indeed, previous work has shown that *loxP* sites with all possible single substitutions at the 4 and 4' positions are able to recombine with wild-type *loxP* sites and with themselves, but less product is observed compared to *loxP* × *loxP* recombination<sup>19</sup>. Given that the identity of the bases in these positions influences which strand is preferentially cleaved to begin recombination and also which strand is preferentially cleaved to resolve the HJ intermediate<sup>35</sup>, we asked whether the various pairings of purines in the 4 and 4' position of *loxP* affect the efficiency of recombination between directly repeated *loxP* sites.

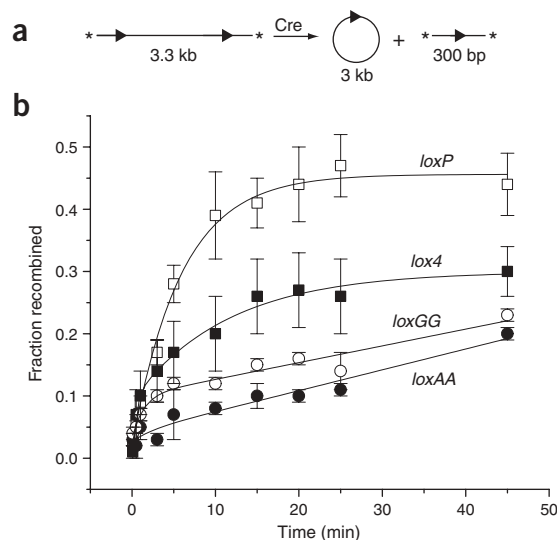
To compare recombination efficiencies, we measured the kinetics of intramolecular recombination between directly repeated *loxP* variant sites on a linearized plasmid (**Fig. 7a**) and compared the results to those obtained for wild-type *loxP*. In addition to *lox4* (where Ade4' and Gua4 are swapped), we tested a *loxP* mutant with Ade in both the

using the same set of recombinases as for *loxP*-TB, we found that in each case the preference had been switched to TS cleavage (**Fig. 6b**). The selectivity had also increased to the extent that we observed almost undetectable BS cleavage by the K201A mutant. The scissile bases in the 4 and 4' positions of *loxP* therefore have a primary role in determining which strand is first cleaved, as suggested earlier on the basis of an analysis of HJ intermediates<sup>16</sup>.

From our analysis of *loxP* cleavage and synapsis, we assumed that the TS-cleavage preference observed for *lox4* was derived from preferred formation of a TS synapse. When we attempted to repeat the FRET experiment shown in **Figure 5** with *lox4*-TF and *lox4*-BF substrates (shown in **Supplementary Fig. 1** online), we did indeed observe more energy transfer for *lox4*-TF relative to *lox4*-BF. However, this experiment was complicated by an unexpected FRET signal for the *lox4*-BF substrate alone, which prevented us from properly comparing the two alternative complexes. The simplest interpretation of this observation is that the *lox4* site is intrinsically bent<sup>36</sup>, and that the bend is in a direction that favors TS-synapse formation (**Fig. 5a**).

The original structural models for the pre-cleavage Cre-*loxS* synaptic complex (*loxS* is a symmetrized variant of *loxP*) indicated that the *lox* site was sharply bent in one half-site and that the opposite half-site was activated for cleavage<sup>30</sup>. A large negative roll was observed at the site of bending, with an opening of the major groove toward the synaptic interface and a corresponding compression of the minor groove toward the Cre-DNA interface. According to this model, bending of the left half-site is associated with BS cleavage (intermediate I in **Fig. 1a**) and bending of the right half-site is associated with TS cleavage. We reasoned that minor-groove compression of the A/T-rich left half-site of *loxP* should be energetically favored relative to the same type of bend in the more G/C-rich right half-site, providing a plausible explanation for why the BS synapse is preferred and the GpC linkage is preferentially cleaved.

To test this idea, we prepared suicide substrates containing a single base change in the *loxP* site, where Ade4' was replaced by 2,6-diaminopurine (DAP). The DAP/T base pair (**Fig. 6a**) has G/C character in the sense that three hydrogen bonds can form, but the functional groups in the major groove remain unchanged relative to the A/T bp. According to the simple half-site bending model described above, the introduction of a 2-amino group in the Ade-4' base would be expected to disfavor minor-groove compression in the left half-site and therefore decrease the bias toward formation of a BS synapse.



**Figure 7** Recombination efficiency of *loxP* variants. **(a)** Schematic of intramolecular excision assay. Arrowheads indicate identical *loxP* (or variant) sites. Products were separated on 5% polyacrylamide gels and quantified. **(b)** Intramolecular recombination of *loxP*-, *lox4*-, *loxGG*-, *loxAA*- and *loxS*-containing substrates by wild-type Cre. Curves are two-exponential fits to the data and do not refer to a specific model.

4' and 4 position (*loxAA*), and the corresponding site with Gua in both positions (*loxGG*). As shown in **Fig. 7b**, the *loxAA*- and *loxGG*-containing plasmids are poor substrates for recombination relative to *loxP*, and the *lox4*-containing substrate shows an intermediate level of efficiency. We have also tested the fully symmetric *loxS* substrate<sup>30</sup> and found that it recombines with efficiency similar to that observed for *loxGG* (data not shown).

For *lox4*, we know that the initial cleavage of substrates is efficient, but on the opposite strand compared to *loxP*. The *loxGG* substrate differs by only one functional group on the purine C6-position relative to the 2,6-diaminopurine-containing *loxP* site tested, which was also cleaved efficiently but on the top strand. These sites are therefore not defective in recombination because they are poorly cleaved relative to *loxP*. As discussed in the following section, an alternative explanation seems more likely, given the role of the modified bases in dictating cleavage preferences and the known preference of Cre to resolve the top strands in the HJ intermediate<sup>35</sup>.

## DISCUSSION

The mechanistic basis for ordered strand exchange in the Cre-*loxP* system has not yet been established<sup>37</sup>. In the experiments described here, we have focused on the relationship between synapsis and cleavage of *loxP* sites by Cre. Using suicide *loxP* substrates containing 5'-bridging phosphorothioates at the cleavage sites, we found that Cre preferentially cleaves the bottom strands of *loxP* under conditions that favor synapsis. In the absence of synapsis, TS cleavage occurs exclusively, but at a lower rate. The cleavage preference of the Cre K201A mutant on singly substituted *loxP* sites, combined with a FRET experiment designed to distinguish between the TS and BS synaptic complexes, strongly support a model in which preferential formation of a BS synapse is what dictates the order of strand exchange in the Cre-*loxP* system.

In proposing that Cre-*loxP* recombination begins with preferential formation of a unique synaptic complex, we are assuming that normal recombination is initiated by cleavage of *loxP* sites within a synaptic complex (shown in **Fig. 1a**). Alternatively, if recombination is initiated by cleavage of unsynapsed *loxP* sites, which then associate to form a complex of pre-formed covalent Cre-*loxP* intermediates, then our data could instead support a mechanism in which the top strands are first exchanged. It seems unlikely, however, that covalent intermediates formed as a result of isolated *loxP* site cleavage would be present a sufficient fraction of the time to be able to encounter a second site and initiate recombination primarily through this mechanism. We would therefore argue that the cleavage preferences observed in the context of the synaptic complex are most relevant to the normal recombination pathway, as has been suggested for FLP recombinase<sup>38</sup>.

An important question to address in considering ordered strand exchange and the role of the scissile bases in the Cre-*loxP* system is whether these mechanistic issues have any bearing on the ability of Cre to recombine *loxP* sites. The available data strongly suggest that asymmetry is indeed an important feature of the Cre-*loxP* system. The efficiency of recombination is reduced by mutations in *loxP* that also change the initial cleavage preference. Previous studies have shown that all single substitutions at the scissile 4 and 4' bases lead to *loxP* sites that recombine less efficiently than wild-type *loxP*, as measured by accumulation of product after a given time using mammalian extracts containing expressed Cre<sup>19</sup>. We reexamined two of these *loxP* mutants as well as the *lox4* mutant for recombination *in vitro* using highly purified Cre. We found that the more symmetric *loxAA* and *loxGG* variants are severely deficient in completing recombination, despite the fact that Ade and Gua are naturally occurring

scissile bases in the *loxP* site. Our attempt to increase the symmetry of *loxP* to include the bases flanking the 6-bp crossover region therefore had a profound negative effect on recombination, suggesting that evolution has fine-tuned this system in subtle ways that involve controlling the reaction through the asymmetric *loxP* spacer.

Many of the mutations that lead to inefficient overall recombination (including *loxAA* and *loxGG*) also lead to an accumulation of Holliday intermediates<sup>19</sup>. A consequence of loss of ordered cleavage in the Cre-*loxP* system may therefore be an efficient but nonproductive cycle of forming and resolving HJ intermediates, with only rare isomerization and resolution of the intermediates to give products. This idea is consistent with the observation that *loxP*-derived HJ substrates are resolved to give primarily TS-cleavage products<sup>35</sup>, which implies that the HJ intermediate is somehow biased to go 'forward' in the pathway. TS cleavage of *loxP* to start the reaction would therefore result in formation of the "favored" HJ-resolution substrate, which may partly explain why modifications to the 4 and 4' position of *loxP* leads to decreased recombination efficiency. The Cre-*loxP* system has apparently evolved to stage the recombination pathway so that the 'less favored' bottom strands are exchanged first, with HJ resolution then favoring product formation<sup>37</sup>.

## METHODS

**Preparation of recombinase proteins and oligonucleotides.** Single-site mutants of Cre were prepared using the Quickchange procedure (Stratagene) starting with wild-type Cre in pET21a (ref. 31). Wild-type and mutant proteins were expressed and purified as native proteins without fusion tags as described<sup>39</sup>. Protein concentrations were measured by UV absorbance at 280 nm, using an extinction coefficient of 49.1 M<sup>-1</sup>cm<sup>-1</sup>, which was experimentally determined as described<sup>40</sup>. Oligonucleotides were synthesized in-house with an Expedite 8900 synthesizer or by the W.M. Keck Facility at Yale University. The protocols used to prepare and purify oligonucleotides containing 5'-bridging phosphorothioates and for FRET experiments are provided in **Supplementary Methods**.

**Cleavage assays.** Ten pmol of oligonucleotide containing a 5'-bridging phosphorothioate was 5'-end labeled with <sup>32</sup>P and annealed to either an unmodified complementary strand (for TS and BS substrates, **Fig. 2a**) or to a labeled complementary strand also containing a bridging sulfur substitution (for TB substrates) by slow cooling in a thermal cycler. Equivalent labeling efficiencies for top and bottom strands of the TB substrates were confirmed by denaturing PAGE analysis of individual strands before annealing and by cleavage of substrates with silver nitrate. Cleavage reactions were performed in a reaction buffer containing 20 mM Sodium HEPES buffer, pH 7.5, 150 mM NaCl, 2 mM DTT, 0.1 mg ml<sup>-1</sup> sonicated Salmon sperm DNA, and 0.1 mg ml<sup>-1</sup> BSA (NCB buffer), with 200 pM radiolabeled DNA substrate plus 40 nM unlabeled, but otherwise identical, substrate. Reactions were initiated by addition of Cre to 400 nM and incubating at 37 °C. Aliquots (20 μl) were taken at appropriate time intervals and quenched by addition of SDS to 1.3% and incubated at 70 °C for 2 min. Samples were digested with proteinase-K at 37 °C for 1 h and analyzed on 10% denaturing polyacrylamide gels. Dried gels were quantified by phosphorimager analysis (Molecular Dynamics). Fractional cleavage was calculated as the amount of product divided by total counts in a lane (for TS and BS sites) or the amount of product divided by 0.5 × total counts (for TB sites, as only one of the two labeled strands can be cleaved).

**FRET synapsis assay.** Fluorescence experiments were performed with a Photon Technology Inc. Quantamaster model C60/2000 L-format scanning spectrofluorometer at 25 °C. Solutions contained 100 nM 54mer labeled DNA duplex and 400 nM Cre K201A or Cre A36V in 10 mM HEPES buffer (pH 7.5), 5 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM DTT, 0.1 mg ml<sup>-1</sup> BSA and 50 μg ml<sup>-1</sup> poly dI/dC DNA and were allowed to equilibrate for 20 min before scanning. Three fluorescence emission spectra were recorded and averaged for each sample from 560 to 700 nm, with an excitation wavelength of 555 nm. Spectra were also recorded for DNA labeled with only the donor and with only the acceptor in the presence and absence of protein and were used to normalize

the dual-labeled spectra. Energy transfer efficiencies ( $E$ ) were calculated as  $E = 1 - (I_{DA}/I_D)$ , where  $I_{DA}$  and  $I_D$  are the normalized donor intensities in the presence and absence of acceptor, respectively<sup>41</sup>.

**Recombination assays.** Substrates for *in vitro* intramolecular recombination were pBluescriptIIISK+ (Stratagene) derivatives containing *loxP* sites (or variants) arranged as direct repeats, with a 300-bp linker between the sites. Restriction digestion at the center of the linker followed by <sup>32</sup>P 5'-end labeling results in a linear substrate for *in vitro* recombination. Recombination assays were carried out at 37 °C in 50 µl volumes containing 50 nM Cre, 500 pM <sup>32</sup>P-labeled substrate, 10 mM bis-tris-propane, pH 7.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mg ml<sup>-1</sup> salmon sperm DNA and 0.1 mg ml<sup>-1</sup> bovine serum albumin. Aliquots (5 µl) were taken at specific time intervals and quenched with SDS to 1% at 70 °C for 2 min followed by proteinase K digestion for 1 h at 37 °C. Samples were analyzed by native PAGE (5%) and quantified with a phosphor-imager. Fractional recombination was calculated for each time point as the amount of product divided by the total counts in a given lane.

Note: Supplementary information is available on the Nature Chemical Biology website.

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Hoess, R.H., Ziese, M. & Sternberg, N. P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. USA* **79**, 3398–3402 (1982).
- Sternberg, N., Hamilton, D., Austin, S., Yarmolinsky, M. & Hoess, R. Site-specific recombination and its role in the life cycle of bacteriophage P1. *Cold Spring Harb. Symp. Quant. Biol.* **45**, 297–309 (1981).
- Nash, H.A. Site-specific recombination: integration, excision, resolution, and inversion of defined DNA segments. in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (ed. Neidhardt, F.C. et al.) 2363–2376 (ASM Press, Washington, D.C., 1996).
- Stark, W.M., Boocock, M.R. & Sherratt, D.J. Catalysis by site-specific recombinases. *Trends Genet.* **8**, 432–439 (1992).
- Azaro, M.A. & Landy, A.  $\lambda$  integrase and the  $\lambda$  Int family. in *Mobile DNA II* (eds. Craig, N.L., Craigie, R., Gellert, M. & Lambowitz, A.M.) 118–148 (ASM Press, Washington D.C., 2002).
- Abremski, K. & Hoess, R. Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J. Biol. Chem.* **259**, 1509–1514 (1984).
- Barre, F.X. et al. FtsK functions in the processing of a Holliday junction intermediate during bacterial chromosome segregation. *Genes Dev.* **14**, 2976–2988 (2000).
- McCulloch, R., Coggins, L.W., Colloms, S.D. & Sherratt, D.J. Xer-mediated site-specific recombination at *cer* generates Holliday junctions in vivo. *EMBO J.* **13**, 1844–1855 (1994).
- Kitts, P.A. & Nash, H.A. Bacteriophage lambda site-specific recombination proceeds with a defined order of strand exchanges. *J. Mol. Biol.* **204**, 95–107 (1988).
- Nunes-Düby, S., Matsumoto, L. & Landy, A. Site-specific recombination intermediates trapped with suicide substrates. *Cell* **50**, 779–788 (1987).
- Arciszewska, L.K. & Sherratt, D.J. Xer site-specific recombination in vitro. *EMBO J.* **14**, 2112–2120 (1995).
- Sauer, B. Chromosome manipulation by Cre-*loxP* recombination. in *Mobile DNA II* (eds. Craig, N.L., Craigie, R., Gellert, M. & Lambowitz, A.M.) 38–58 (ASM Press, Washington D.C., 2002).
- Van Duyn, G.D. A structural view of Cre-*loxP* site-specific recombination. *Annu. Rev. Biophys. Biomol. Struct.* **30**, 87–104 (2001).
- Van Duyn, G.D. A structural view of tyrosine recombinase site-specific recombination. in *Mobile DNA II* (eds. Craig, N.L., Craigie, R., Gellert, M. & Lambowitz, A.M.) 93–117 (ASM Press, Washington DC, 2002).
- Hoess, R., Wierzbicki, A. & Abremski, K. Isolation and characterization of intermediates in site-specific recombination. *Proc. Natl. Acad. Sci. USA* **84**, 6840–6844 (1987).
- Lee, L. & Sadowski, P.D. Sequence of the *loxP* site determines the order of strand exchange by the Cre recombinase. *J. Mol. Biol.* **326**, 397–412 (2003).
- Martin, S.S., Pulido, E., Chu, V.C., Lechner, T.S. & Baldwin, E.P. The order of strand exchanges in Cre-*LoxP* recombination and its basis suggested by the crystal structure of a Cre-*LoxP* Holliday junction complex. *J. Mol. Biol.* **319**, 107–127 (2002).
- Ennifar, E., Meyer, J.E., Buchholz, F., Stewart, A.F. & Suck, D. Crystal structure of a wild-type Cre recombinase-*loxP* synapse reveals a novel spacer conformation suggesting an alternative mechanism for DNA cleavage activation. *Nucleic Acids Res.* **31**, 5449–5460 (2003).
- Lee, G. & Saito, I. Role of nucleotide sequences of *loxP* spacer region in Cre-mediated recombination. *Gene* **216**, 55–65 (1998).
- Krogh, B.O. & Shuman, S. Catalytic mechanism of DNA topoisomerase IB. *Mol. Cell* **5**, 1035–1041 (2000).
- Burgin, A.B., Jr., Huizenga, B.N. & Nash, H.A. A novel suicide substrate for DNA topoisomerases and site-specific recombinases. *Nucleic Acids Res.* **23**, 2973–2979 (1995).
- Burgin, A.B. & Nash, H.A. Suicide substrates reveal properties of the homology-dependent steps during integrative recombination of bacteriophage lambda. *Curr. Biol.* **5**, 1312–1321 (1995).
- Burgin, A.B. & Nash, H.A. Symmetry in the mechanism of bacteriophage lambda integrative recombination. *Proc. Natl. Acad. Sci. USA* **89**, 9642–9646 (1992).
- Xu, Y. & Kool, E.T. A novel 5'-iodonucleoside allows efficient nonenzymatic ligation of single-stranded and duplex DNAs. *Tetrahedron Lett.* **38**, 5595–5598 (1997).
- Miller, G.P. & Kool, E.T. A simple method for electrophilic functionalization of DNA. *Org. Lett.* **4**, 3599–3601 (2002).
- Ringrose, L. et al. Comparative kinetic analysis of FLP and Cre recombinases: mathematical models for DNA binding and recombination. *J. Mol. Biol.* **284**, 363–384 (1998).
- Abremski, K., Wierzbicki, A., Frommer, B. & Hoess, R.H. Bacteriophage P1 Cre-*loxP* site-specific recombination. Site-specific DNA topoisomerase activity of the Cre recombinase protein. *J. Biol. Chem.* **261**, 391–396 (1986).
- Wierzbicki, A., Kendall, M., Abremski, K. & Hoess, R. A mutational analysis of the bacteriophage P1 recombinase Cre. *J. Mol. Biol.* **195**, 785–794 (1987).
- Cheng, C., Kussie, P., Pavletich, N. & Shuman, S. Conservation of structure and mechanism between eukaryotic topoisomerase I and site-specific recombinases. *Cell* **92**, 841–850 (1998).
- Guo, F., Gopaul, D.N. & Van Duyn, G.D. Asymmetric DNA bending in the Cre-*loxP* site-specific recombination synapse. *Proc. Natl. Acad. Sci. USA* **96**, 7143–7148 (1999).
- Guo, F., Gopaul, D.N. & Van Duyn, G.D. Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. *Nature* **389**, 40–46 (1997).
- Gopaul, D.N., Guo, F. & Van Duyn, G.D. Structure of the Holliday junction intermediate in Cre-*loxP* site-specific recombination. *EMBO J.* **17**, 4175–4187 (1998).
- Ghosh, K., Lau, C.K., Guo, F., Segall, A.M. & Van Duyn, G.D. Peptide trapping of the Holliday junction intermediate in Cre-*loxP* site-specific recombination. *J. Biol. Chem.* **280**, 8290–8299 (2005).
- Sherratt, D.J. & Wigley, D.B. Conserved themes but novel activities in recombinases and topoisomerases. *Cell* **93**, 149–152 (1998).
- Lee, L. & Sadowski, P.D. Directional resolution of synthetic holliday structures by the Cre recombinase. *J. Biol. Chem.* **276**, 31092–31098 (2001).
- Lee, L., Chu, L.C. & Sadowski, P.D. Cre induces an asymmetric DNA bend in its target *loxP* site. *J. Biol. Chem.* **278**, 23118–23129 (2003).
- Lee, L. & Sadowski, P.D. Strand selection by the tyrosine recombinases. in *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 80 (ed. Moldave, K.) 1–42 (2005).
- Voziyanov, Y., Lee, J., Whang, I. & Jayaram, M. Analyses of the first chemical step in FLP site-specific recombination: synapsis may not be a pre-requisite for strand cleavage. *J. Mol. Biol.* **256**, 720–735 (1996).
- Ghosh, K. & Van Duyn, G.D. Cre-*loxP* biochemistry. *Methods* **28**, 374–383 (2002).
- Gill, S.C. & von Hippel, P.H. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326 (1989).
- Clegg, R.M. Fluorescence resonance energy transfer and nucleic acids. *Methods Enzymol.* **211**, 353–388 (1992).
- Hoess, R.H., Wierzbicki, A. & Abremski, K. The role of the *loxP* spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14**, 2287–2300 (1986).