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# Recombination of Hybrid Target Sites by Binary Combinations of Flp Variants: Mutations that Foster Interprotomer Collaboration and Enlarge Substrate Tolerance

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Strategies of directed evolution and combinatorial mutagenesis applied to the Flp site-specific recombinase have yielded recombination systems that utilize bi-specific hybrid target sites. A hybrid site is assembled from two half-sites, each harboring a distinct binding specificity. Satisfying the two specificities by a binary combination of Flp variants, while necessary, may not be sufficient to elicit recombination. We have identified amino acid substitutions that foster interprotomer collaboration between partner Flp variants to potentiate strand exchange in hybrid sites. One such substitution, A35T, acts specifically in *cis* with one of the two partners of a variant pair, Flp(K82M) and Flp(A35T, R281V). The same A35T mutation is also present within a group of mutations that rescue a Flp variant, Flp(Y60S), that is defective in establishing monomer–monomer interactions on the native Flp target site. Strikingly, these mutations are localized to peptide regions involved in interdomain and interprotomer interactions within the recombination complex. The same group of mutations, when transferred to the context of wild-type Flp, can relax its specificity to include non-native target sites. The hybrid Flp systems described here mimic the naturally occurring XerC/XerD recombination system that utilizes two recombinases with distinct DNA binding specificities. The ability to overcome the constraints of binding site symmetry in Flp recombination has important implications in the targeted manipulations of genomes.

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## Introduction

The Flp protein encoded by the 2 μm yeast plasmid, as well as other members of the tyrosine family site-specific recombinases, carries out recombination within a tetrameric protein assembly bound to two DNA partners.[1,2] In the majority of cases, four identical subunits constitute the active recombinase. In a rare instance, exemplified by the *Escherichia coli* XerC/XerD system and its homologs in other bacteria, the active entity is a pair of heterodimers formed by two distinct recombinase subunits (XerC and XerD).[3] The "simple" members of the family such as Flp and the Cre protein of phage P1 require only a pair of DNA targets and four protein monomers to assemble the recombination synapse. The more "complex" members (lambda Int and XerC/XerD, for example) may utilize rather elaborate interactions involving accessory DNA sites and protein factors to organize a similar synapse.[3,4]

The core DNA site for tyrosine family recombination consists of two recombinase binding elements arranged in head-to-head orientation on either side of a strand exchange region (or spacer). In the case of the native Flp target site (*FRT*), the two 13 bp binding elements are identical except for 1 bp (position 2; see Figure 1), and flank an

Abbreviations used: FRT, Flp recombination target; mFRT, mutant FRT; hmFRT, hybrid mFRT.

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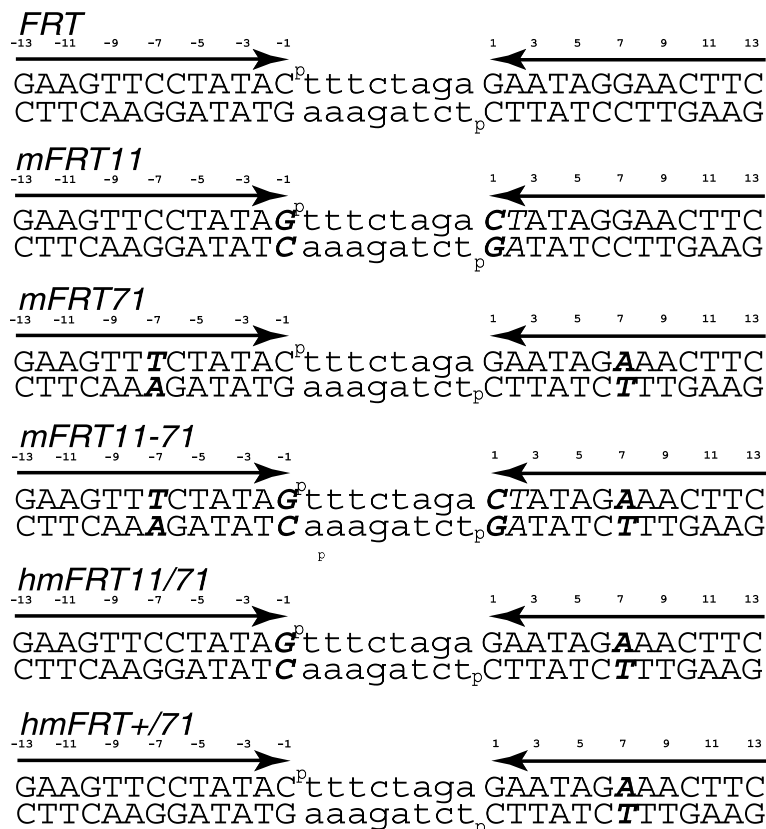
(a)




(b)

| Flp / Flp variant | Recombination activity | | | |
|---|---|---|---|---|
| | *FRT* | *mFRT11* | *mFRT71* | *mFRT11-71* |
| **Flp** | **Active** | - | - | - |
| **Flp*11(K82Y)** | **Active** | **Active** | - | - |
| **Flp*11(K82M)** | **Active** | **Active** | - | - |
| **Flp*71(R281V)** | - | - | **Active** | - |
| **Flp*[71, 11-71] (A35T, K82M, R281V)** | - | - | **Active** | **Active** |

**Figure 1**. Mutant *FRT* sites and altered specificity Flp variants. (a) The native *FRT* site with the two head-to-head Flp binding elements and the included strand exchange region (spacer DNA) is shown at the top. The scissile phosphate groups are indicated as p on the top and bottom strands. The altered base-pairs in *mFRT11*, *mFRT71* and *mFRT11-71*, conferring shifts from native specificity, are written as bold characters in italics. As explained,[13,14] the single asymmetric base-pair in the Flp binding element of *FRT* (position 2) is symmetrized in *mFRT11* and *mFRT11-71* by changing it in the right binding element. The new base-pair is shown in italics. In *mFRT71* and in the hybrid target sites (*hmFRTs*), the asymmetry at position 2 is retained as in *FRT*. (b) A subset of the previously isolated Flp variants and their cognate target sites, as determined by *in vivo* and *in vitro* recombination assays,[13,14] are tabulated.

8 bp spacer. Flp is a monomer in solution, and binds to each binding element as a monomer. The two monomers interact with each other by introducing a DNA bend within the spacer[5,6] to establish a dimer interface. Synapsis of two Flp bound DNA partners sets the stage for triggering the two coordinated strand cleavages that initiate recombination. One round of recombination is completed in two temporally distinct steps. First, the exchange of one pair of strands results in a Holliday junction intermediate, which is then resolved into reciprocal recombinants by the exchange of the second pair of strands.

Based on a number of biochemical studies and structural information from free recombinase proteins and proteins complexed with cleaved or uncleaved DNA substrates or with a synthetic Holliday junction, a unified picture of the recombination pathway for the tyrosine family can be inferred.[1–4,7] The synaptic complex is essentially planar, with the DNA sites placed in an antiparallel alignment. The complex has a perfect 2-fold and an approximate 4-fold symmetry, such that two of the monomer–monomer interfaces are catalytically functional and the other two are non-functional. Hence only two of the potential four active sites are in their active state, and only one pair of strands can be broken and exchanged. Following formation of the Holliday intermediate, the complex isomerizes to silence the first pair of active sites and activate the second pair. Depending on the length of the spacer, isomerization may involve a simple scissoring of the DNA arms or a more complex reconfiguration of the complex. The latter includes not only scissoring but also slight movement of the arms into or below the junction plane. Concomitantly, the protein subunits undergo modest rotational as well as translational motions. The newly activated pair of active sites then completes recombination by exchanging the second pair of strands.

The confluence of the solution and crystallographic data reveal critical conditions that must be

satisfied for the chemistry of recombination to be productively orchestrated within the synaptic complex. First, each binding site must be correctly recognized by the associated recombinase subunit in order to orient four specific phosphodiester bonds for cleavage. Second, allosteric interactions among the four DNA-bound recombinase subunits must activate/inactivate the appropriate pairs of active sites during Holliday junction formation and resolution. This exquisite catalytic regulation is achieved by establishing a cyclic relay of contacts between adjacent protein monomers. A particular consequence of such monomer–monomer interactions in the case of Flp, but not the other well-characterized members of the tyrosine family, is the assembly of a shared active site at the dimer interface.[8,9]

Recombinase variants with new binding specificities are potentially useful in probing how target recognition initiated by DNA–protein contacts is channeled into the catalytic steps of recombination *via* intra- and interprotomer signaling. Such variants have been obtained for both Flp and Cre by applying strategies for directed evolution.[10–14] The recombination properties of a subset of the Flp variants on mutant *FRT* sites containing base-pair substitutions at position 1 (*mFRT11*), at position 7 (*mFRT71*) or at positions 1 and 7 (*mFRT11-71*) reveal relaxed specificities in some cases and more stringent novel specificities in others[14] (Figure 1(b)).

Here, we have employed the action of Flp variant pairs on hybrid *mFRT* sites as a novel strategy to examine interpartner collaboration during site-specific recombination. The analysis has revealed amino acid substitutions in Flp that potentiate such collaboration. Interestingly, one of these mutations (A35T) is functional only when present in *cis* with one partner of a Flp variant pair acting on a certain hybrid target site. In corollary experiments, we identified a set of mutations (A35 belongs to this group) that can rescue a Flp variant that is defective in establishing a functional dimer interface on *FRT*. The same amino acid changes, introduced in otherwise wild-type Flp, relax its normally stringent target specificity. Based on the crystal structure of the Flp–DNA complex,[6] the peptide regions that house these mutations are likely involved in promoting functional interactions within a Flp monomer and/or between neighboring Flp monomers in response to DNA recognition. The hybrid recombination systems, together with the possibility of constructing Flp variants that recognize multiply altered binding sites,[14] open potential new avenues in the application of site-specific recombination for genome engineering.

## Results

### Flp variants with evolved novel specificities

*In vitro* evolution by mutagenesis and gene shuffling, combined with *in vivo* screening in *E. coli*, have revealed Flp variants that have acquired recombination competence on target sites containing altered Flp binding elements[13,14] (Figure 1). The variant *FRT* sites were assembled by incorporating base-pair alterations at position 1 or 7 or both positions in the left and right Flp binding elements to yield *mFRT11*, *mFRT71* and *mFRT11-71*, respectively[14] (Figure 1(a)). For the work reported here, additional hybrid *mFRT* sites were derived by combining half-sites from *FRT* as well as the three primary *mFRT*s. For example, *hmFRT11/71* refers to a site with a left binding element containing the first position mutation (11) and the right binding element containing the seventh position mutation (71). When the left–right disposition of the same two binding elements is switched, the resulting site is *hmFRT71/11*. Similarly, *hmFRT+/71* contains a native binding element at the left, whereas the binding element at the right harbors the seventh position mutation.

A subset of the Flp protein variants relevant to the present study and their target preferences, inferred from *in vivo* and *in vitro* recombination assays, are arranged in Figure 1(b). A Flp variant, marked by an asterisk, is usually denoted by a generic name that identifies its acquired novel specificity. For example, Flp*11 stands for a variant or a set of variants that can recombine *mFRT11*; similarly, Flp*[71, 11-71] indicates a variant that can act on *mFRT71* and *mFRT11-71*. When referring to a given member of a certain specificity class, the amino acid substitution(s) harbored by it are listed in parentheses. For example, Flp*11(K82M) and Flp*11(K82Y) are both active on *mFRT11*.

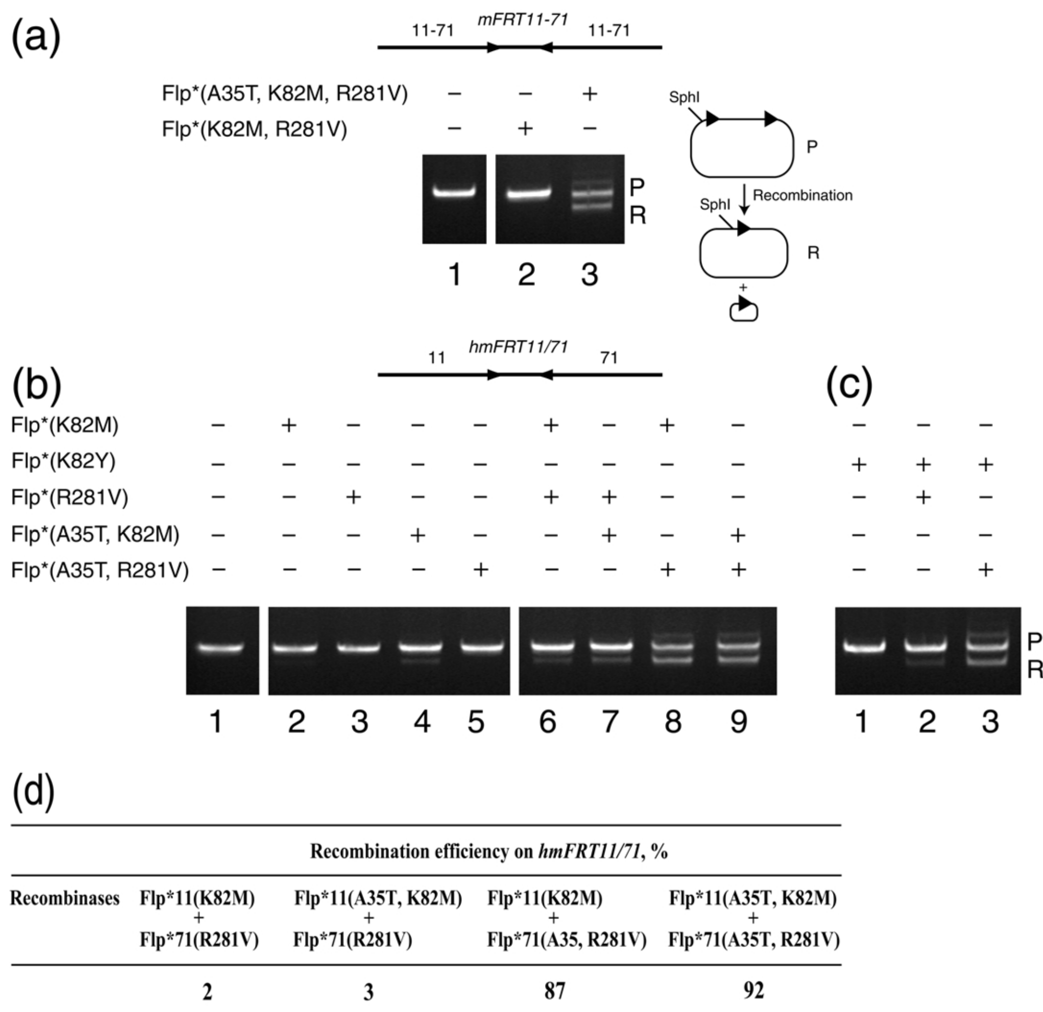
Results from a series of binding reactions with *FRT*, *mFRT11*, *mFRT71* and *mFRT11-71* (data not shown) suggest that the recombination specificities of the tested Flp variants have evolved primarily at the level of DNA binding. This finding is consistent with positions 1 and 7 of *FRT* being important contact points for Flp: position 1 being contacted by Lys82 and position 7 by Arg281.[6]

### Action of a binary combination of Flp variants on a hybrid *mFRT* site: an accessory mutation A35T acts in *cis* with one of the Flp variant partners to potentiate recombination

In previous work,[14] our attempt to obtain an active recombinase for *mFRT11-71* (doubly mutated at base-pair positions 1 and 7 in each half-site) by combining the 11-specific mutation K82M and the 71-specific mutation R281V in Flp*(K82M, R281V) was unsuccessful, as determined by the *in vivo* deletion test. However, we discovered that a single additional mutation A35T can substantially improve recombination of *mFRT11-71 in vivo* by Flp*(A35T, K82M, R281V). This triple variant of Flp can also utilize *mFRT71*, and has hence been abbreviated as Flp*[71, 11-71] (see Figure 1(b)). We show in Figure 2(a) the effect of A35T on *in vitro* recombination of *mFRT11-71* to provide a frame of reference

for similar reactions of a hybrid site containing 11 and 71 specificities. In a deletion reaction utilizing head-to-tail repeats of *mFRT11-71*, Flp*(K82M, R281V) did not show detectable recombination activity (Figure 2(a), lane 2). By comparison, Flp*(A35T, K82M, R281V) converted over one-third of the input substrate into the deletion product (R) (Figure 2(a), lane 3). We next examined how the A35T mutation, with its strong influence on *mFRT11-71* recombination, affects the recombination outcome from a hybrid site containing the two specificities, but in separate half-sites. The results from a set of reactions carried out with *hmFRT11/71* are shown in Figure 2(b).

The individual Flp variants, Flp*11(K82M), Flp*71(R281V), Flp*(A35T, K82M) and Flp*(A35T, R281V) gave no recombination or extremely weak recombination on *hmFRT11/71* (Figure 2(b), lanes 2–5). Even when an equimolar mixture of Flp*11(K82M) and Flp*71(R281V) was provided in the reaction, recombination was barely detectable (Figure 2(b), lane 6) Similarly, when the reaction contained Flp*11(A35T, K82M) and Flp*71(R281V) as the partners, the recombination efficiency was quite low (Figure 2(b), lane 7). However, recombination was greatly stimulated when Flp*71(A35T, R281V) was paired with Flp*11(K82M) or with Flp*11(A35T, K82M) (Figure 2(b), lanes 8 and 9). The *in vitro* results agreed with the outcomes of *in*




| Recombination efficiency on *hmFRT11/71*, % | | | | |
|---|---|---|---|---|
| Recombinases | Flp*11(K82M) + Flp*71(R281V) | Flp*11(A35T, K82M) + Flp*71(R281V) | Flp*11(K82M) + Flp*71(A35, R281V) | Flp*11(A35T, K82M) + Flp*71(A35T, R281V) |
| | 2 | 3 | 87 | 92 |

**Figure 2**. The A35T mutation acts in *cis* with Flp*71(R281) to promote functional collaboration between Flp*71(A35T, R281V) and Flp*11(K82Y) or Flp*11(K82M) during recombination of *hmFRT11/71*. Results of recombination reactions with *mFRT11-71* are shown in (a) and those with *hmFRT11/71* in (b) and (c). The relevant differences between the two sites are indicated in their representations at the top. The intramolecular deletion reactions, shown schematically in (a), were carried out between two directly repeated copies of the recombination target present on a circular plasmid. The reactions were analyzed after digestion with *Sph* I, which cuts once within the parental plasmid P and the larger of the two deletion circles R. The smaller deletion circle (~500 bp), which migrates much farther during electrophoresis, is not shown here. (d) The results of an *in vivo E. coli* assay[13] for the protein and substrate combinations corresponding to lanes 6–9 of B are tabulated. Here, recombination excises the *lacZ*α reporter cassette bordered by two direct copies of the recombination target site. The recombination efficiency is expressed as a ratio of white colonies to the sum of blue and white colonies, averaged from three experiments. The standard deviation was approximately 5% of the mean for all assays.

*vivo* deletion assays using the corresponding Flp variant combinations (Figure 2(b), compare lanes 6–9 to Figure 2(d)).

Does the combined A35T-R281V effect persist when Flp*71(A35T, R281V) is paired with a Flp variant other than Flp*11(K82M) that also has 11 specificity? The assays shown in Figure 2(c) were done by substituting Flp*11(K82Y) for Flp*11(K82M). Flp*11(K82Y) alone or the Flp*11-(K82Y)/Flp*71(R281V) combination was either practically inactive (no R band was detected in lane 1; Figure 2(c)) or was highly incompetent (only a faint R band was seen in lane 2; Figure 2(c)) in *hmFRT11/71* recombination. However, with the combination of Flp*11(K82Y) and Flp*71(A35T, R281V), the deletion product was readily detected (Figure 2(c), lane 3).

The *hmFRT11/71* reactions presented in Figure 2(b) demonstrate that satisfying each half-site specificity of the hybrid site by a binary mixture of Flp variants (R281V for the 71 half-site and K82M for the 11 half-site, for example) is not sufficient to accomplish recombination. However, addition of the A35T mutation in Flp*71(R281V), allows the double mutant Flp*71(A35T, R281V) to pair with Flp*11(K82M) and recombine *hmFRT11/71*. At the same time, inclusion of A35T in Flp*11-(K82M) is not productive, or only weakly so, in stimulating recombination when Flp*71(R281V) lacks this mutation. The presence of A35T in both protein partners is only as good as having it in the Flp*71(R281V) variant alone. Thus, for this particular substrate and Flp variant combination, the stimulatory effect of A35T requires the mutation to be in *cis* with R281V. However, the effect of the A35T/R281V combination of mutations is not specific for Flp(K82M) alone. As shown in Figure 2(c), it is also felt by Flp*11(K82Y), which has a similar target specificity as Flp*11(K82M). The present results are consistent with the previous finding that all three mutations, A35T, K82M and R281V, need to be present simultaneously in a Flp variant for it to be able to recombine *mFRT11-71* (containing base-pair changes at positions 1 and 7 in both binding elements)[14] (Figure 2(a), compare lanes 2 and 3).

### A mutation at a DNA contacting Flp residue, K82M, can promote partner collaboration during recombination of *hmFRT11/71*

The results of the assays shown in Figure 2(b)–(d) prompted us to ask whether mutations other than A35T might also mediate partner activation during recombination of *hmFRT11/71*. Preliminary *in vitro* screens using binary combinations of Flp variants that specify 11 and 71 half-site binding specificities indicated that Flp(K82M, R281V) together with Flp(K82Y) or Flp(K82M) can utilize this hybrid site in recombination. As detailed in Figure 3, the abilities of these Flp variant pairs to mediate intramolecular and intermolecular strand exchange in *hmFRT11/71* were explored further.

In the intramolecular deletion assay with *hmFRT11/71*, Flp*11(K82Y) and Flp*(K82M, R281V) alone gave no detectable recombinant products (absence of "R" in lanes 2 and 3; Figure 3(a)). However, in a reaction containing an approximately equimolar mixture of the two proteins, recombination was readily apparent (Figure 3(a), lane 4). Although a trace amount of the deletion product was formed with Flp*11(K82M) alone (Figure 3(a), lane 5), the reaction was far more robust when it was paired with Flp*(K82M, R281V) (Figure 3(a), lane 6). The weak band migrating just above the parental band (P) in lanes 4 and 6 of Figure 3(a) was likely due to a low level of intermolecular recombination.

Does the interprotomer collaboration between Flp*(K82M, R281V) and, say, Flp*11(K82Y) work in intermolecular reactions as well? The assays shown in Figure 3(b) test recombination between a circular plasmid containing a copy of *hmFRT11/71* and a linear fragment derived from it that also includes this site. The primary recombination event between the two molecules will coalesce them into a linear product. Further iterated rounds of recombination will generate a series of products (R) that can be visualized as a ladder of recombinant bands by agarose gel electrophoresis. As in the intramolecular reactions, recombination by Flp*(K82M, R281V) or Flp*11(K82Y) alone was too weak to be detected (Figure 3B, lanes 1 and 7, respectively). By contrast, reactions containing a mixture of the two proteins showed a large stimulation in recombination activity (Figure 3(b), lanes 2–6). Essentially identical results were obtained with *hmFRT71/11* (Figure 3(c)) as well as with *hmFRT+/71* and *hmFRT71/+* (data not shown).

The results from Figure 3 reveal that combining the K82M and R281V substitutions enables Flp(K82M, R281V) to collaborate effectively with Flp(K82Y) or Flp(K82M) to perform *hmFRT11/71* recombination. In other words, in this substrate context, K82M behaves functionally analogous to A35T, although they may bring about this common effect by different mechanisms. Based on the individual binding specificities provided by the K82Y (or K82M) and the R281V mutations,[13,14] the data are consistent with the active recombinase pair being constituted by Flp(K82Y) or Flp(K82M) bound to the 11 half-site and Flp(K82M, R281V) bound to the 71 half-site. Hence, the proposed binding pattern was subjected to further verification as described below (Figure 4, and the relevant text).

### Cleavage complementation between Flp*11(K82Y) and Flp*(K82M, R281V) derivatives on *hmFRT11/71* defines the half-site occupancy of each protein

Two Flp monomers bound to each half-site of an *FRT* target assemble a cleavage pocket by the donation of the nucleophile Tyr343 from one monomer to the pro-active site (that includes

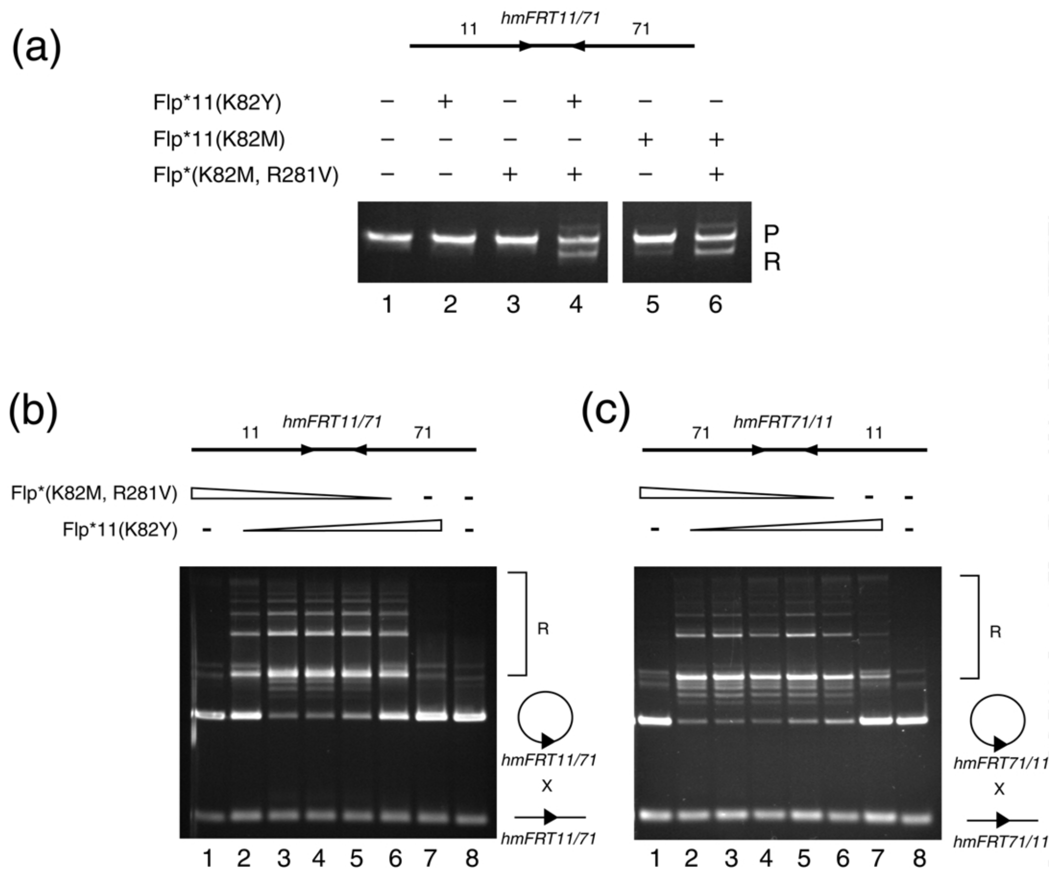



**Figure 3**. Collaboration between Flp*(K82M, R281V) and Flp*11(K82Y) or Flp*11(K82M) during recombination of *hmFRT11/71* and *hmFRT71/11*. The hybrid sites used for the assays in (a)–(c) are shown schematically above the respective panels. (a) The intramolecular deletion reactions were carried out and analyzed as described for Figure 2. (b) and (c) Intermolecular recombination reactions were performed between a circular plasmid and a linear fragment, each harboring *hmFRT11/7* in one case (b), and *hmFRT71/11* in the other (c). The ladder of recombinants resulting from iterated rounds of reaction are collectively represented as R.

Arg191, Lys223, His305, Arg308 and Trp330) of the second monomer.[1,7,9] A Flp(Y343F)-Flp(R191S) dimer occupying *FRT* can only cleave the scissile phosphate group adjacent to Flp(Y343F) using Tyr343 delivered by Flp(R191S). Hence, the position of strand cleavage in a catalytic complementation reaction can unequivocally identify the half-site with which a given Flp monomer is associated. In the assays shown in Figure 4(a), the substrate was *hmFRT11/71*, and the Flp variants were derivatives of Flp*11(K82Y) and Flp*(K82M, R281V) mutated at Tyr343 or Arg191. For simplicity, these proteins are referred to below as Flp* or Flp** (the single asterisk denoting the K82Y substitution and double asterisks denoting the K82M plus R281V substitutions), respectively, and the particular active site mutation in each case is indicated in parentheses.

The 105 bp long *hmFRT11/71* hybrid site was labeled at the 3′ end on both strands, so that products from breakage of the scissile phosphate proximal to the 11 binding site at the left or that proximal to the 71 binding site at the right could be monitored simultaneously. When analyzed by electrophoresis under denaturing conditions, the length of the former would be 68 nt and that of the latter 38 nt. In a reaction containing an equimolar mixture of Flp* and Flp**, cleavage occurred at the left and right ends. Note that there was an intrinsic bias, estimated by densitometry to be approximately 3 to 1, that favored cleavage at the left end ($C_L$ in lane 1; Figure 4(a)) over that at the right end ($C_R$ in lane 1; Figure 4(a)). This inequality was not due to a difference in the labeling efficiencies of the two strands. The bands resulting from the digestion of the substrate with *Xba* I (which cuts within the spacer) were more or less similar in their intensities (Figure 4(a), lane X). Despite this disparity in strand cleavage by Flp, the results from the complementation test were clear-cut. For the Flp** (R191S) plus Flp* (Y343F) combination, cleavage was prominent at the left end ($C_L$ in lane 2; Figure 4(a)), whereas cleavage at the right end was almost undetectable ($C_R$ in lane 2; Figure 4(a)). From overexposed autoradiograms, to obtain at least semi-quantitative integration of the extremely weak lower band, the ratio of cleavage at the left to that at the right was determined to be at least 10 to

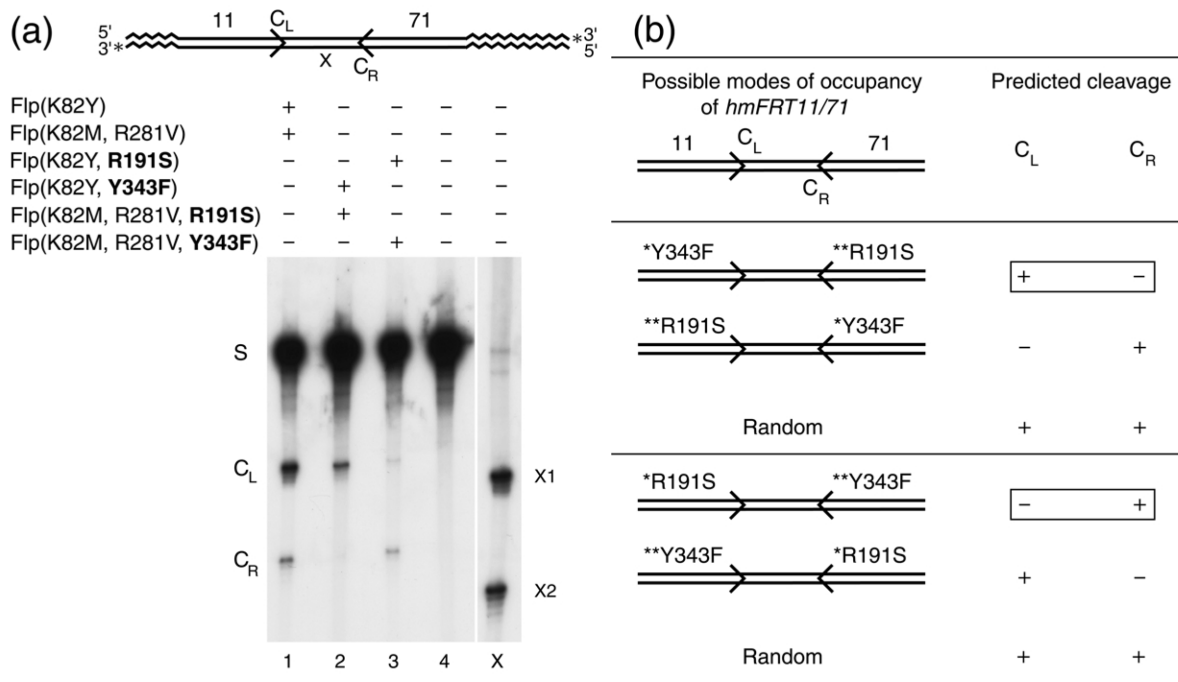



**Figure 4**. Cleavage complementation between catalytic mutant derivatives of Flp*11(Y82) and Flp*(K82M, R281V) on *hmFRT11/71*. (a) The two active site mutants derived from Flp*11(K82Y) contained R191S and Y343F substitutions, and are designated in the text as Flp*(R191S) and Flp*(Y343F), respectively. The equivalent derivatives of Flp*(K82M, R281V) are referred to in the text as Flp** (R191S) and Flp** (Y343F), respectively. For completeness, all of the mutations present in a Flp variant are spelled out here, and the relevant active site mutations are indicated in bold. In the schematic of the substrate, the asterisks represent the 3′ end label, $C_L$ and $C_R$ the cleavage points adjacent to the 11 and 71 half-sites, respectively, and X marks the *Xba*I site within the spacer. Cleavage reactions were analyzed by electrophoresis in 10% denaturing polyacrylamide. The labeled strands of the substrate are called S. (b) The possible modes of occupancy of *hmFRT11/71* by the binary Flp variants used in lanes 2 and 3 of (a) and the predicted cleavage products are tabulated. The experimentally observed results are enclosed in boxes.

1. Or, with this pair of proteins, cleavage adjacent to the 11 half-site was increased at least three- to fourfold relative to the control reaction in lane 3. For the reverse combination, Flp** (Y343F) and Flp*(R191S), cleavage was biased towards the right end over the left end by a factor of approximately 2 (Figure 4(a), lane 3). Or, under this situation, there was a roughly sixfold preference in cleavage (taking into account the threefold natural bias in the opposite direction) adjacent to the 71 half-site as compared to the control reaction.

The possible modes of occupancy of *hmFRT11/71* by the two binary combinations of the active site mutants tested and the corresponding predicted cleavage patterns are tabulated in Figure 4(b). The experimentally observed outcomes best match the predictions enclosed in the boxes. They are consistent with the 11 half-site being almost always occupied by the Flp* derivatives Flp*11(K82Y, Y343F) or Flp(K82Y, R191S) and the 71 half-site by the Flp** derivatives Flp*(K82M, R281V, Y343F) and Flp(K82M, R281V, R191S). Recall that, during cleavage complementation, cleavage must occur adjacent to the Y343F mutant, since it is the recipient of Tyr343.

The sum of the results from Figures 3 and 4 suggests that, in the context of *hmFRT11/71*, the binding specificity of K82M in Flp(K82M, R281V) is recessive to that of R281V. Rather, in its *cis* configuration with R281V, K82M appears to serve an effector function (much like the A35T mutation).

### A35T is one among the set of mutations that potentiates Flp(Y60S) for recombination on native *FRT*

Tyr60 of Flp corresponds to an invariant residue among the Flp-related yeast site-specific recombinases identified so far. Mutations of this position (Y60S, for example) lead to a severe drop in recombination efficiency.[15,16] Flp(Y60S) binds less strongly than Flp to *FRT*, and two bound monomers of this mutant cannot induce the same degree of the spacer centered DNA bend as a wild-type Flp dimer. However, Flp(Y60S) is able to carry out efficient strand cleavage in an *FRT* site that is prebent with the help of an A3 or A5 nucleotide bulge in the spacer.[17] In addition, it can mediate both strand cleavage and joining in half-*FRT* substrates, containing a single binding element with an adjacent scissile phosphate group and a primarily single-stranded spacer, with comparable efficiency to wild-type Flp.[16] The structural

constraints posed by a full-length double-helical spacer appear to impede the functional dimerization of Flp(Y60S), at least partly due to its weaker DNA binding. In the crystal structure of the Flp–DNA complex, Tyr60 protrudes from helix B to contact the phosphate group between bases 3 and 4 opposite the scissile phosphate group on the non-cleaved strand of a half-site.[6]

Based on the finding that certain amino acid substitutions in Flp can cause partner activation (see Figures 2 and 3), we wished to know whether the effect of the Y60S mutation can be suppressed by one or more additional mutations that promote intersubunit cooperation. The strategy for this search consisted in random mutagenesis of Flp(Y60S) DNA, identification of recombination positive clones by an *in vivo* screen using *FRT* as the substrate[13,14] and subsequent repeated rounds of DNA shuffling, mutagenesis and screening carried out on a pool consisting of the putative positives and Flp(Y60S). By this procedure, we obtained derivatives of Flp(Y60S) that were able to recombine *FRT* with vastly better efficiencies than the parent protein (Figure 5(a)). The most robust of these, Flp(Y60S)$^{sup3}$, was nearly 85% as active as wild-type Flp. The *in vitro* activity of Flp(Y60S)$^{sup3}$ was as expected from the *in vivo* result (Figure 5(b)). One striking point is that the three gain of function variants obtained from Flp(Y60S) harbored A35T, among others, as a common mutation even though the original input DNA did not contain this mutation (or for that matter any of the other sup$^3$ mutations).

We then transferred the sup$^3$ group of mutations to wild-type Flp to test what effects they might have when position 60 is Tyr rather than Ser. In the *in vivo* assay, the recombination activity of Flp$^{sup3}$ on *FRT* was similar to that of Flp (Figure 5(c)). However, in sharp contrast to Flp, Flp$^{sup3}$ yielded relatively high activity on *mFRT11*, modest activities on *mFRT71* and *hmFRT11/71* and weak but detectable activity on *mFRT11-71 in vivo* (Figure 5(c)). *In vitro* assays also revealed the recombination of *mFRT11*, *mFRT71* and *hmFRT11/71* by Flp$^{sup3}$; however, *mFRT11-71* recombination was not detected (Figure 5(d)). Flp(Y60S)$^{sup3}$, on the other hand, was not active on any of the *mFRT*s tested in the *in vivo* (Figure 5(d)) or *in vitro* (data not shown) assays.

The pack of mutations, including A35T, that counteracts the defective activation of catalysis in *FRT* bound Flp(Y60S) maps to peptide regions that can potentially influence the coupling between DNA binding by Flp and activation of catalysis (elaborated in Discussion).

## Discussion

How does a site-specific recombinase recognize its target DNA with exquisite selectivity? And how does it discriminate against non-target sites, even when they are closely related in sequence to the target site? How does functional DNA recognition modulate interactions within and between recombinase monomers so as to configure the active sites for strand cleavage and exchange? The action of altered specificity variants of Flp on hybrid target sites has a direct bearing on these basic issues, and on those concerning practical applications of Flp in genetic engineering.

### Recombination in hybrid *mFRT* sites: amino acid substitutions that promote functional collaboration between Flp variants

We have shown in previous studies[13,14] that the point mutations K82M (or K82Y) and R281V in Flp are responsible for recognition of the 11 and 71 half-sites, respectively, in altered *FRT* sites (or *mFRT*s). Here, we have demonstrated that hybrid *mFRT* sites (*hmFRT11/71* or *hmFRT71/11*) can be recombined by providing pairs of Flp variants that together satisfy their two half-site specificities: Flp(K82M) or Flp(K82Y) for 11 specificity and Flp(K82M, R281V) or Flp(A35T, R281V) for 71 specificity (Figures 2 and 3). A catalytic complementation assay reveals that, despite the binding specificity of K82M for the 11 half-site, Flp(K82M, R281V) facilitates recombination of *hmFRT11/71* by binding only to the 71 half-site (or expressing R281V specificity; Figure 4).

It is not clear how an amino acid change at a DNA-contacting residue (K82M) and one at a non-contacting residue (A35T) present in *cis* with R281V can both enable Flp*(K82M, R281V) or Flp*(A35T, R281V) to partner effectively with Flp(K82M) or Flp(K82Y) to potentiate *hmFRT11/71* recombination. The crystal structures of wild-type Flp and Cre in association with their target sites,[6,18] suggest that DNA recognition triggers allosteric activation, *via* intersubunit interactions, of the pair of active sites for the first strand exchange reaction. It is conceivable that distinct mutations that alter DNA contacts by the recombinase or modulate the subsequent activation step can manifest a common effector function during recombination. The location of Ala35 in the three-dimensional structure of Flp is in agreement with such a notion (see below). Furthermore, structural data on altered specificity variants of Cre underscore the relative ease with which a multiplicity of functionally relevant protein–DNA interaction networks might arise due to macromolecular flexibility and induced fit.[19]

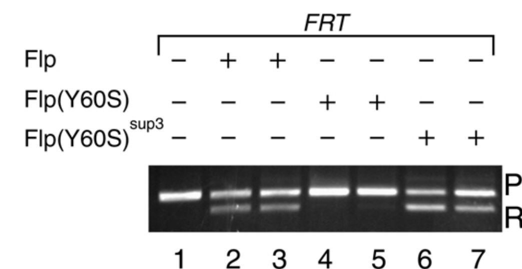
### Hybrid *mFRT* recombination by Flp variants: analogy to the XerC/XerD system

As already mentioned, the tyrosine recombinases normally make use of four subunits of a single protein to carry out recombination. An exception to this rule among the well-characterized members of this family is the *E. coli* XerC/XerD system, and by inference, its homologs in other bacteria. They resolve post-replication dimers (or

(a)

| Flp variant | Amino acid substitutions | | | | | | | | | | | Recombination, % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Flp(Y60S) | | | | | | Y60S | | | | | | <1 |
| Flp(A35T, Y60S) | A35T | | | | | Y60S | | | | | | 1 |
| Flp(Y60S)$^{sup1}$ | A35T | I45V | T50A | | | Y60S | E150G | K173R | I295F | T324I | I367L | 57 |
| Flp(Y60S)$^{sup2}$ | A35T | I45V | | A55H | S59G | Y60S | E150G | K173R | I295F | T324I | I367L | 49 |
| Flp(Y60S)$^{sup3}$ | A35T | I45V | T50A | A55H | S59G | Y60S | E150G | K173R | I295F | T324I | I367L | 83 |

(b)




(c)

| Recombinase | Recombination efficiency, % | | | | |
|---|---|---|---|---|---|
| | *FRT* | *mFRT11* | *mFRT71* | *mFRT11-71* | *hmFRT11/71* |
| Flp | 97 | <0.1 | <0.1 | <0.1 | <0.1 |
| Flp$^{sup3}$ | 98 | 89 | 53 | 4 | 64 |
| Flp(Y60S)$^{sup3}$ | 83 | <0.1 | <0.1 | <0.1 | <0.1 |

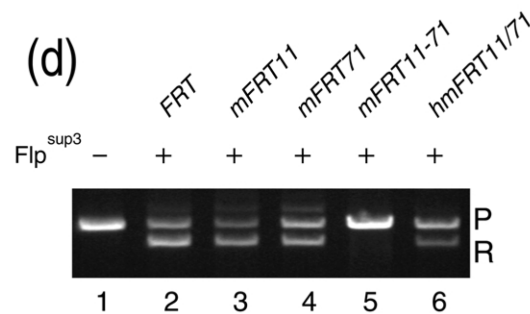



**Figure 5**. Mutations that suppress the recombination defect of Flp(Y60S) on *FRT* also relax the target specificity of wild-type Flp. The deletion assays were carried out *in vivo* ((a) and (c)) as described for Figure 2. The recombination substrate was *FRT* in (a) and *FRT* or the indicated *mFRT*s in (c). The *in vitro* deletion reactions ((b) and (d)) were done as outlined in the legends to Figures 2 and 3. The reactions in (b) were done with approximately four monomers (left lane) or six monomers (right lane) of the indicated protein per half-site. For reactions in (d), the protein to half-site molar ratio was maintained as roughly 4 : 1.

higher oligomers) of bacterial chromosomes and plasmids resulting from homologous recombination into monomers for faithful segregation into daughter cells.[3] In the recombination target site for XerC/XerD, the strand exchange region is flanked by the C specific binding element on one side and the D specific binding element on the other. This naturally occurring bi-specificity, in conjunction with the available structural information for the tyrosine family members, has made it possible to target mutations to peptide regions that are predicted to promote interprotomer collaboration.[20–22] Two interesting classes of such mutations have been thus unveiled using Holliday junction substrates. One causes catalytic activation of the partner recombinase while at the same time impeding catalysis by self. The second class shows the opposite phenotype: self-activation with concomitant impairment of catalysis by the partner. The implication is that the native system has evolved to effect the sequential activation of each recombinase in the two-step strand exchange process.

Whereas amino acids required for reciprocal control in the XerC/XerD system map to peptide regions housing the catalytic tyrosine as well as other active site residues of the respective recombinases and to their extreme C-terminal regions, the effector mutations identified here for *hmFRT11/71* recombination by Flp variants are located in the N-terminal domain, and therefore cannot be directly involved in self or partner activation/inhibition. However, because of the strong interconnectivity of the DNA bound recombinase monomers, the effect of even a distant mutation may be propagated to an active site.

To our knowledge, reactions of the Flp variants described here represent the only case outside of the XerC/XerD system in which a binary protein combination is able to carry out efficient recombination on bi-specific target sites. The related, yet distinct specificities of the λ and HK202 Int proteins have been used to effect DNA cleavage in synthetic Holliday junctions whose arms display these specificities.[23] However, a complete recombination reaction starting with two hybrid DNA partners has not been reported by the combined action of these two recombinases.

### Mutations that suppress defective interprotomer collaboration during recombination of native *FRT*

The unveiling of point mutations that facilitate partner collaboration during recombination of hybrid target sites encouraged us to seek similar suppressor mutations that rescue non-productive interprotomer interactions during reactions of native *FRT*. As exemplified with Flp(Y60S), which cannot establish the normal Flp dimer interface when bound to *FRT*,[15,17] such mutations can indeed be found (Figure 5). In this instance, a set of mutations, rather than a single mutation, that maps to different peptide regions of Flp is responsible for the suppression. Furthermore, the extent of suppression can be different, as assayed by *in vivo* recombination, depending upon the particular combinations of mutations.

It is noteworthy that A35T is common to the set of three Y60S suppressors listed in Figure 5(a), even though A35T by itself has little or no suppressor function (Figure 5(a), row 2). The differences in the recombination activities elicited by the different mutant combinations suggest that it is the sum of the contributions made by the point mutations as a whole that determines the final level of suppression. For example, the absence of A55H and S59G from the sup3 group (called sup1 in row 3; Figure 5(a)) or T50A (called sup2 in row 4; Figure 5(a)) leads to an approximately two-fold drop in recombination efficiency in the *in vivo* deletion assay (Figure 5(a)). When position 35 is restored from Thr to Ala in Flp(Y60S)$^{sup3}$ by directed mutagenesis, the *in vivo* recombination yield decreases by a factor of roughly 3 (data not shown). Another point of interest is that K173R, a member of the sup$^{3}$ class, was also isolated by Buchholz *et al.*[24] in their screen for more thermostable versions of Flp.

### Mutations that restore recombination of *FRT* by Flp(Y60S) likely affect intra- and interprotomer communication

The set of ten mutations that together yield the highest level of suppression of the Y60S recombination defect map to functionally important peptide regions within the N and C-terminal regions of Flp. As revealed by the crystal structure of the Flp–DNA complex,[6] the two domains make contacts between each other within a monomer, and also mediate intermonomer contacts: the former to form a ring around the DNA and the latter to arrange the cyclic configuration of the recombination competent Flp tetramer.

One subset of the suppressor mutations maps to the extreme N-terminal portion of Flp, at positions 35, 45, 50, 55 and 59 (Figure 6(a) and (b)). The first three are clustered in a region that, in wild-type Flp, contacts helix D within the polypeptide linker (residues 114 through 129) tethering the N and C-terminal domains of an adjacent monomer. Ala55 and Ser59 of Flp, in turn, are in contact with the peptide segment housing Ala35, Ile45 and Thr50. The two also make water-mediated DNA contacts within the Flp binding element proximal to the scissile phosphate group, but on the opposite helical face from it.

The second subset of the suppressor mutations, at positions 150, 173, 295, 324 and 367, map to the C-terminal domain of Flp. In wild-type Flp, these residues (except for 173) are located in regions where the C-terminal domains of adjacent Flp monomers interact (Figure 6(a) and (c)) to present the Tyr343 nucleophile from helix M of one Flp subunit (green in Figure 6(c)) to the active site

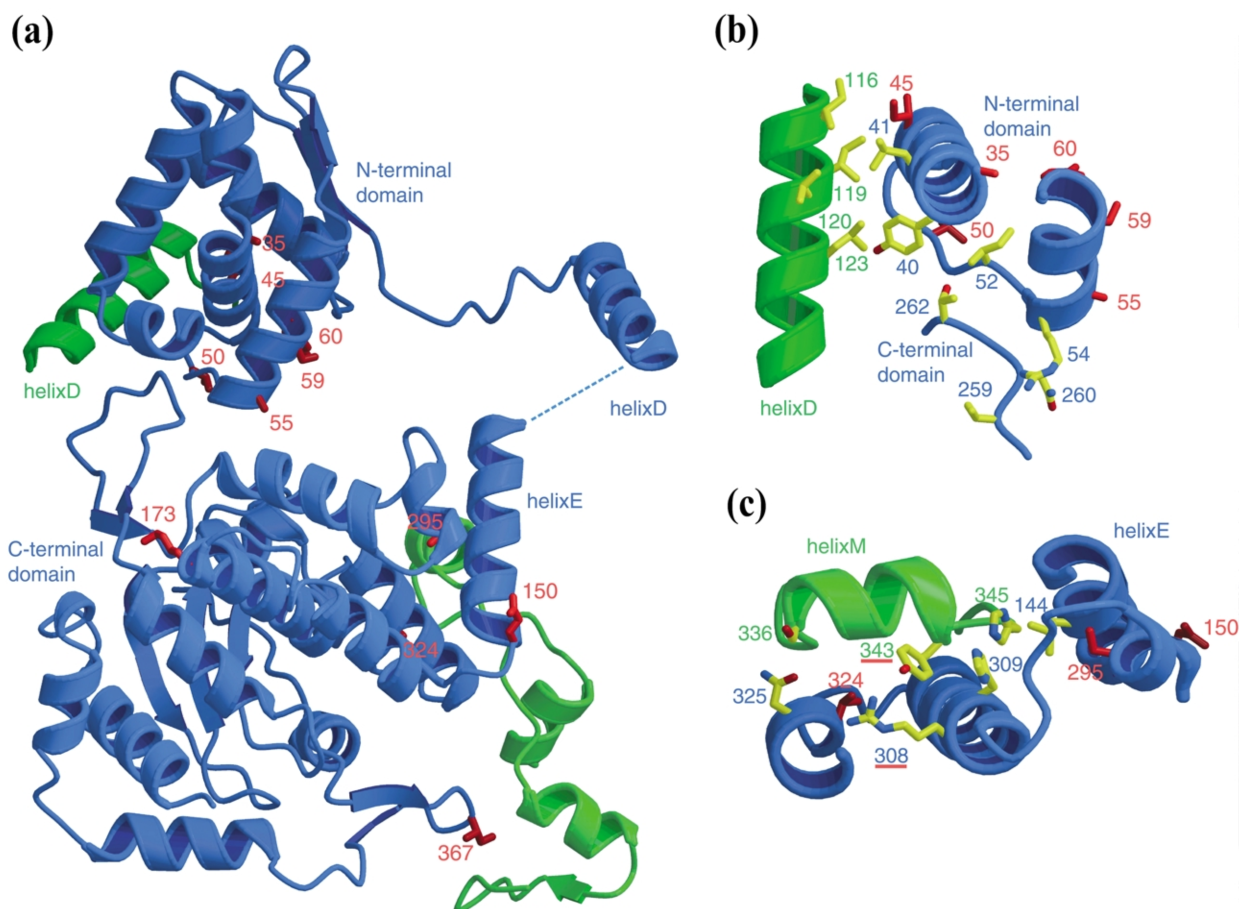



**Figure 6**. Structural implications of the amino acid substitutions that rescue Flp(Y60S) and relax Flp specificity. (a) The Flp monomer shown in blue corresponds to the B subunit in the crystal structure of the protein tetramer bound to DNA in a recombination complex.[6] The amino acid residues whose alterations lead to the sup3 phenotype (restoration of Flp(Y60S) recombination activity on *FRT*) are shown in red. The peptide regions from the two neighboring Flp monomers are shown in green. The Flp structure was analyzed using Swiss-PdbViewer 3.7 (Guex *et al.*).[32] Images of Flp were prepared using BobScript[33,34] and Raster3D.[35] (b) An isolated view of the region where the N-terminal domain of the blue Flp monomer interacts with the same domain of its neighbor as well as its own C-terminal domain is presented. Mutations A35T, I45V, T50A, A55H and S59G form one subset of the sup3 group, and their locations are indicated by marking the corresponding wild-type residues in red. The Tyr60 residue whose substitution by Ser leads to the recombination defect that is suppressed by sup3 is also shown in red. (c) In this view, one of the two regions of interaction between the C-terminal domains of two Flp neighbors is highlighted. The E150G, I295F, T324I mutations that constitute the second subset of the sup3 group are highlighted by showing the corresponding wild-type residues in red. Two of the key catalytic residues in Flp (Y343 and R308) are underlined in red. For reasons of clarity, not all of the amino acid interactions are displayed in (b) and (c). Hydrogen bonds and DNA are also omitted.

cleft of the neighboring Flp subunit (blue in Figure 6(c)). The amino acid residues 295 and 324 also contribute to the hydrogen bonding and hydrophobic interactions that encompass the active site residues in a "catalytic net".[14] The effect of position 173 on functional target recognition by Flp (or Flp(Y60S)) is likely to be indirect. Helix G harboring Lys173 contacts helix J, which makes several direct DNA contacts in the middle part of the Flp binding element. In addition, helix G contacts the base of a fold-back peptide loop that sticks out towards and interacts with the N-terminal domain (Figure 6(a) and (b)).

Although the combined effects of the ensemble of the Y60S suppressor mutations can be reasonably accommodated by their locations, structural information is essential to provide a more rational basis for their function. Recent structural studies on Cre variants bound to their target sites reveal that DNA flexibility, protein side-chain shifts and establishment of water-mediated contact networks offer multiple possibilities for recognition as well as discrimination of DNA target sites by proteins.[19] Amino acid alterations that do not make direct DNA contact or partake in subunit interactions may still restructure DNA–protein or protein–protein interfaces in ways that are difficult to predict.

### Suppressors of the Y60S mutation increase tolerance of altered target sites by Flp: implications in the progressive evolution from multi to mono-specificity

The collection of mutations that suppress the recombination defect of Flp(Y60S) also relax the target specificity of wild-type Flp, enabling it to act on *mFRT11*, *mFRT71*, *hmFRT11/71* and even on *mFRT11-71*, albeit quite weakly on the last target site. It is likely that the early progenitor of Flp, perhaps derived from a type IB topoisomerase, was a more promiscuous recombinase that, over evolutionary time, narrowed its activity to the unique specificity of present day Flp. One reliable means of ensuring mono-specificity is to block the assembly of an active recombination complex whenever an inappropriate DNA substrate is encountered. The bidomainal organization of Flp and the four-subunit architecture of the recombinase are well suited for readjusting intra- and intermolecular interactions following DNA contact. It is thus feasible to effect stable and functional association with a cognate site and rapid dissociation from a non-cognate one. Amino acid changes that affect this "proof reading" step can slow down the rejection of a non-substrate DNA sequence, and induce relaxed specificity.

### Implications of hybrid site recombination by Flp in targeted genome rearrangements

The intrinsic simplicity of the Flp and Cre recombination reactions and the relative ease of functionally expressing these proteins in a variety of biological systems have made them popular tools in the targeted manipulation of genomes.[25–27] Strategies of directed evolution combined with appropriate screening and selection schemes have made it possible to obtain altered specificity variants of these recombinases.[10–14] Furthermore, our previous work has demonstrated the feasibility of creating, *via* DNA shuffling and mutagenesis, Flp variants with combinatorial changes in specificity from those harboring specificity to single base-pair changes in the Flp binding element.[14] Recombination of hybrid *mFRT*s by Flp variant mixtures and the relaxation of Flp target specificity by a finite set of mutations documented here mark another important step in the application of site-specific recombination as a genetic engineering tool. Normally, recombination occurs on target sites with symmetric or nearly symmetric recombinase binding elements with identical specificity, although the spacer sequence can be quite variable. The probability of random occurrence of such a site is extremely low even in large genomes. Hence, the target site has to be first engineered into a genetic locus before it can be manipulated by the cognate recombinase. The ability to overcome the symmetry barrier, to induce relaxed specificity in Flp (or Flp variants) and to mediate recombination in target sites with multiple alterations in the binding elements can, in principle, greatly improve the chances of identifying and utilizing naturally occurring sequences within a genome for bringing about desired DNA rearrangements.

## Materials and Methods

### Purification of Flp and Flp variants

The Flp protein and its variants were purified using described protocols.[28] The affinity purification step using an oligo-*FRT* column was omitted. Instead, a final step of adsorption to a 15 S column (BioRad; Hercules, CA) at 0.15 M NaCl followed by washes of 0.2 M, 0.27 M and 0.37 M NaCl, was employed. At 0.37 M NaCl, the bound Flp was stripped from the column. The purity of the individual preparations was approximately 70–80%.

### *In vitro* recombination reactions

The *in vitro* recombination assays were performed using more or less the same conditions as described earlier[29] with approximately four monomers of Flp (or Flp variant) per Flp binding element. The reactions, each containing approximately 2 μg of the plasmid substrate, were incubated in the recombination buffer (25 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 5% (w/v) polyethylene glycol (PEG) 6000) for 30 minutes at 30 °C. Following heat inactivation of the recombinase at 80 °C for ten minutes, samples were made 0.1% (w/v) in SDS, and treated with proteinase K for 30 minutes at 37 °C. DNA was recovered using Qiagen PCR purification kit for analysis by agarose gel electrophoresis. For deletion assays, DNA samples were digested with either HindIII or SphI prior to electrophoresis.

### *In vivo* recombination assays

*In vivo* recombination assays in *E. coli* were based on the excision of a *lacZ*α reporter cassette, and were carried out as described for testing single Flp variants.[13] For testing the action of a binary combination of Flp variants on hybrid *mFRT* sites, the bacterial cells harbored two compatible plasmids, derivatives of pBAD33[30] and pBBR1MSC2,[31] from which the proteins were expressed by arabinose induction for 2.5 hours. The reporter plasmid in the single and double variant assays was a derivative of pBAD24,[30] and was compatible with either of the expression plasmids.

### DNA cleavage assays

Strand cleavage was performed as described[8,9] on an approximately 100 bp EcoRI–BamHI DNA fragment. The 3′ end of each strand was labeled by the "fill in" reaction using Klenow polymerase and all four [α-$^{32}$P]dNTPs. After incubation of the labeled substrate with the appropriate Flp variant mixtures at 30 °C for 30 minutes, reactions were stopped by the addition of SDS (0.2% final concentration) and treated with proteinase K (20 μg per reaction) for one hour at 37 °C. DNA was recovered from each reaction by phenol/chloroform extraction and ethanol precipitation. Samples were

fractionated by electrophoresis in 10% (w/v) denaturing polyacrylamide gels (acrylamide/bis-acrylamide, 19 : 1).

### DNA mutagenesis and shuffling

Error-prone mutagenesis and DNA shuffling were done essentially as described.[24]

### Other methods

The Flp–DNA structure was analyzed using Swiss PDB Viewer 3.7.[32]

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