SUPPLEMENTAL MATERIAL

Table S1. I-DmoI Constructs

Designation[1]	**Class**[2]	**Description**
I-DmoI$_{CL}$	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, F109Y, K111A, L113F, V115D, A116G
I-DmoI$_{CLD}$	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, F109Y, K111A, L113F, V115D, A116G, E117D
I-DmoI$_{CLE}$	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, D20E, F109Y, K111A, L113F, V115D, A116G
I-DmoI$_{CLED}$	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, D20E, F109Y, K111A, L113F, V115D, A116G, E117D
CL$_{G116A}$	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, F109Y, K111A, L113F, V115D
CL$_{D115V}$	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, F109Y, K111A, L113F, A116G
CL$_{D19I}$	LAGLIDADG	Amino acids changes: L15A, L17F, F109Y, K111A, L113F, V115D, A116G
CL$_{G116A,D115V}$ (I-DmoI$_{H}$)	LAGLIDADG	Amino acids changes: L15A, L17F, I19D, F109Y, K111A, L113F
CL$_{G116A,D19I}$	LAGLIDADG	Amino acids changes: L15A, L17F, F109Y, K111A, L113F, V115D
CL$_{G116A,D115V,D19I}$	LAGLIDADG	Amino acids changes: L15A, L17F, F109Y, K111A, L113F
CL$_{D115V,D19I}$	LAGLIDADG	Amino acids changes: L15A, L17F, F109Y, K111A, L113F, A116G
I-DmoI$_{098/099}$	Split[3]	Sequence order: A, B; Domain A, protein residues 1-98; Domain B, protein residues 99-194
I-DmoI$_{098/101}$	Split	Sequence order: A, B; Domain A, protein residues 1-98; Domain B, protein residues 101-194
I-DmoI$_{098/103}$	Split	Sequence order: A, B; Domain A, protein residues 1-98; Domain B, protein residues 103-194
I-DmoI$_{098/105}$	Split	Sequence order: A, B; Domain A, protein residues 1-98; Domain B, protein residues 105-194
I-DmoI$_{100/099}$	Split	Sequence order: A, B; Domain A, protein residues 1-100; Domain B, protein residues 99-194
I-DmoI$_{100/101}$	Split	Sequence order: A, B; Domain A, protein residues 1-100; Domain B, protein residues 101-194
I-DmoI$_{100/103}$	Split	Sequence order: A, B; Domain A, protein residues 1-100; Domain B, protein residues 103-194
I-DmoI$_{100/105}$	Split	Sequence order: A, B; Domain A, protein residues 1-100; Domain B, protein residues 105-194
I-DmoI$_{102/099}$	Split	Sequence order: A, B; Domain A, protein residues 1-102; Domain B, protein residues 99-194
I-DmoI$_{102/101}$ (I-DmoI$_{S}$)	Split	Sequence order: A, B; Domain A, protein residues 1-102; Domain B, protein residues 101-194
I-DmoI$_{102/103}$	Split	Sequence order: A, B; Domain A, protein residues 1-102; Domain B, protein residues 103-194
I-DmoI$_{102/105}$	Split	Sequence order: A, B; Domain A, protein residues 1-102; Domain B, protein residues 105-194
I-DmoI$_{104/099}$	Split	Sequence order: A, B; Domain A, protein residues 1-104; Domain B, protein residues 99-194
I-DmoI$_{104/101}$	Split	Sequence order: A, B; Domain A, protein residues 1-104; Domain B, protein residues 101-194
I-DmoI$_{104/103}$	Split	Sequence order: A, B; Domain A, protein residues 1-104; Domain B, protein residues 103-194

I-DmoI$_{104/105}$	Split	Sequence order: A, B; Domain A, protein residues 1-104; Domain B, protein residues 105-194
I-DmoI$_{101/102}$ (I-DmoI$_{SS}$)	Split	Sequence order: B, A; Domain B, protein residues 101-194; Domain A, protein residues 1-102
I-DmoI ($_f$W)	Epitope	I-DmoI with N-terminal FLAG epitope
I-DmoI (W$_c$)	Epitope	I-DmoI with C-terminal *c-myc* epitope
I-DmoI ($_f$W$_c$)	Epitope	I-DmoI with N-terminal FLAG and C-terminal *c-myc* epitopes
I-DmoI$_S$ ($_f$S)	Epitope	I-DmoI$_S$ with FLAG epitope on the N-terminus of domain A protein
I-DmoI$_S$ (S$_c$)	Epitope	I-DmoI$_S$ with *c-myc* epitope on the C-terminus of domain B protein
I-DmoI$_S$ ($_f$S$_c$)	Epitope	I-DmoI$_S$ with FLAG epitope on the N-terminus of domain A protein and *c-myc* epitope on the C-terminus of domain B protein
I-DmoI$_{SS}$ (SS$_c$)	Epitope	I-DmoI$_{SS}$ with *c-myc* epitope on the C-terminus of domain A protein

[1]Designation in parentheses represents the common name used in the text
[2]Mutant classes: LAGLIDADG, amino acid changes in the LAGLIDADG helices; Split, constructs comprised of separate domain A and B proteins; Epitope, constructs containing a FLAG epitope (amino acids DYKDDDDK) and/or *c-myc* epitope (amino acids EQKLISEEDL) as noted
[3]Residue numbers in split construct proteins (A and B) correspond to residue positions in full-length I-DmoI

Figure S1. DNA mobility-shift assay. (1) no-protein control, (2) I-DmoI, (3) I-DmoI$_{CL}$, (4) I-DmoI$_{H}$ and (5) I-DmoI$_{S}$. The proportion of bound DNA correlates (±10%) with the relative cleavage activity of each construct as noted in Figure 4B. BC, bound-complex; UB, unbound DNA; bands above BC are non-specific lysate artifacts.

Figure S2. Potential interactions of the I19D and V115D mutations in the I-DmoI$_{CL}$ LAGLIDADG interface. (**A**) The LAGLIDADG interface of I-CreI. The interactions of the naturally occurring D18 and D18′ are shown. Left, Cα trace of the I-CreI homodimer with the area of interest boxed and pertinent residues in green. Right, detailed view of boxed region. Hydrogen bonding is observed with residues within the interface (OH of Y12′ and Y12) and with backbone NHs of residues in the subunit loop (K96, L97, K98 and K96′, L97′, K98′). Interactions are indicated by thin lines with distances shown in Å. (**B**) The modeled LAGLIDADG interface of I-DmoI$_{CL}$. Theoretical interactions for the D19 and D115 mutations are shown. Left, Cα trace of the I-DmoI monomer with the area of interest boxed and pertinent residues in green. Right, detailed view of boxed region. Unlike the homodimeric I-CreI, potential interactions for the aspartic acids differ on each side of the LAGLIDADG helices in I-DmoI. D19 is positioned to interact with the existing N129 and with the newly introduced F109Y change of the CL interface. Neither of these interactions appears disruptive but rather would serve to satisfy hydrogen bonding potential for the newly introduced chemical groups in D19 and Y109. In contrast, D115 not only has the potential to disrupt the Y13/H51 interaction but could also interfere with K120, which by structural analogy to K98 in I-CreI, K403 in PI-SceI, K104 in I-MsoI and K122 in I-SceI (7,8,10,11,16) is presumed to form part of the active site.

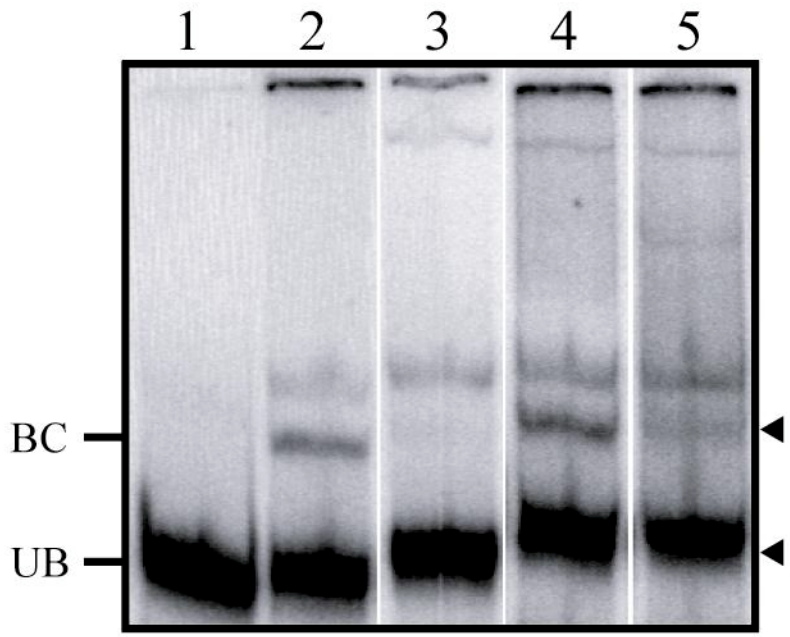

Figure S1.

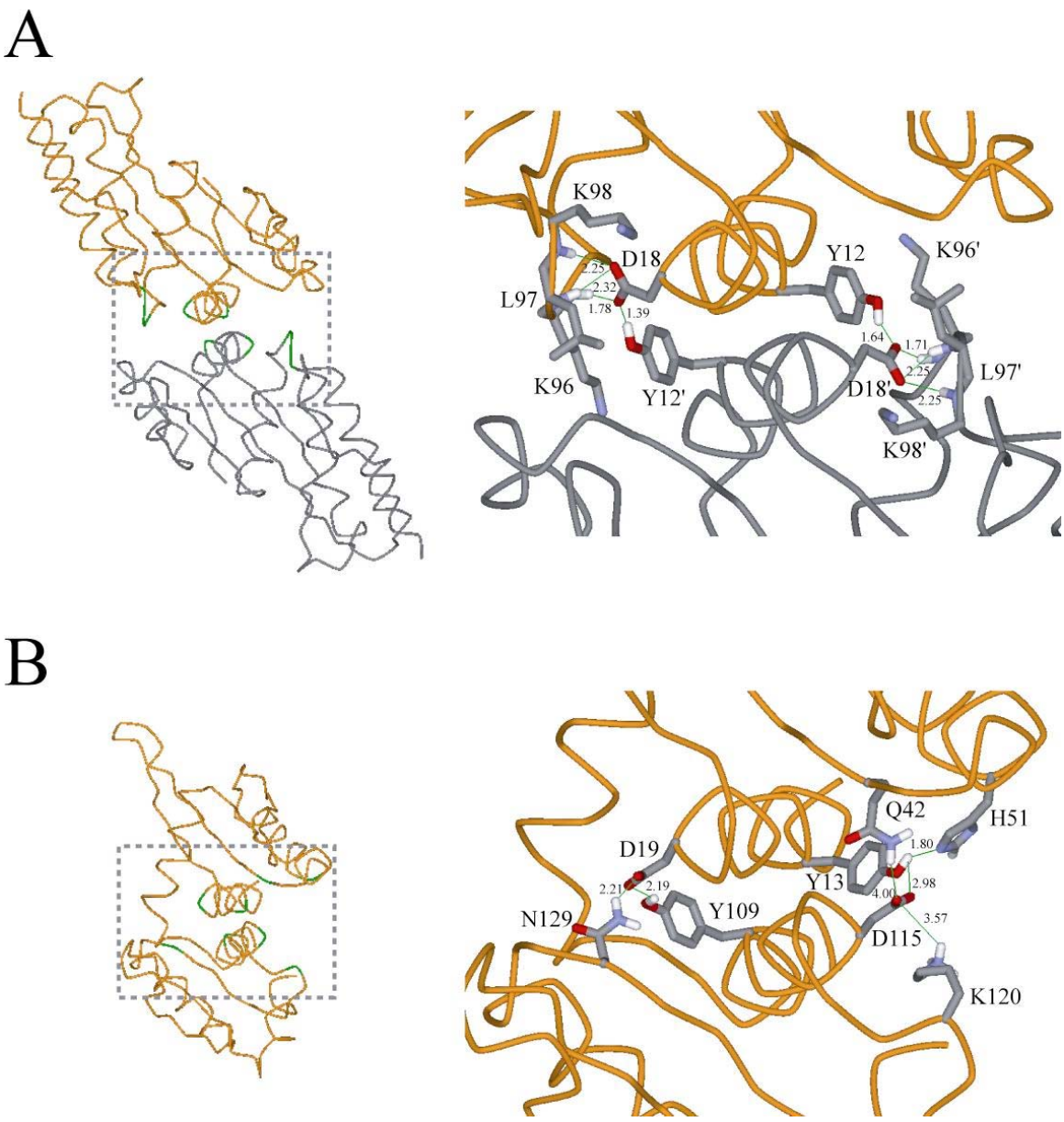
A
K98
D18
Y12
K96'
L97
2.25
2.32
1.78
1.39
1.64
1.71
2.25
2.25
L97'
K96
Y12'
D18'
K98'
B
Q42
H51
D19
1.80
Y13
2.98
2.21
2.19
4.00
N129
Y109
3.57
D115
K120

Figure S2.