Table S1. Sequences of Meganucleases in this Study

(with Formal and Abbreviated Construct Names; **related to all Figures and Tables**)

I-SmaMI
SKGENSKLNPWAVVGFIDAEGSFMVRVRKNSKYKTGWLVVAIFSVTVDKKDLFLLESLKTFFGGLGSIKKSGNSTFSYRIESSEQLTKIILP
FFDKYSLITEKLGDYLLFKKVLELMGTKEHLTQRGLEKIVSLKASINKGLSEELQAAFPQCVPTPRPEINNKLIPDPFWLAGFVSGDGSFKSI
LKKSESIKVGFQSILVFQITQHARDVKLMESLISYLGCGFIEKDSRGPWLYYTVTNFSDIQGKIIPFFHQYKIIGSKYGDYMDWCKIALIMQN
KNHLTPEGLNEIRALKGGMNKGRL

I-SmaMI-e-hGATA (eSmaGATA)
SKGENSKLNPWAVVGFIDAEGSFMVRVRKRSDMKTGWLVTAIFSVGVDKKDLFLLESLKTFFGGLGSIKKAGNSVFLYRIYSSEQLTKIIL
PFFDKYSLITEKLGDYLLFKKVLELMGTKEHLTQRGLEKIVSLKASINKGLSEELQAAFPQCVPTPRPEINNKNIPDPFWLAGFVSGDGSFK
SYLTKAEWSKVGFQSHLEFQITQHARDVKLMESLISYLGCGYISKDSRGPWLYYRVTRFSDIQGKIIPFFHQYKIIGSKYGDYNDWCKIALI
MQNKNHLTPEGLNEIRALKGGMNKGRL

I-OnuI
SRRESINPWILTGFADAEGSFLLRIRNNNKSSVGYSTELGFQITLHNKDKSILENIQSTWKVGVIANSGDNAVSLKVTRFEDLKVIIDHFEKY
PLITQKLGDYMLFKQAFCVMENKEHLKINGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVNLIKSKS
KLGVQVQLVFSITQHIKDKNLMNSLITYLGCGYIKEKNKSEFSWLDFVVTKFSDINDKIIPVFQENTLIGVKLEDFEDWCKVAKLIEEKKHLT
ESGLDEIKKIKLNMNKGR

I-OnuI-e-Therm (eOnuTherm)
SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHFEKY
PLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVNLIKSKS
KLGVQVQLVFSITQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTE
EGLDEIKKIKLNMNKGR

I-OnuI-e-ThermE178D (eOnuTherm-E178D)
SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHFEKY
PLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGDGCFFVNLIKSKS
KLGVQVQLVFSITQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTE
EGLDEIKKIKLNMNKGR

I-OnuI-e-Therm-bCtxA (eOnuTherm-bCtxA)
SINPWILTGFADAEGSFGLRIRKRNKSSVGYSTELGFEIKLHNKDKSILENIQSTWGVGVIANSGSNAVRLRVTRFEDLKVIIDHFEKYPLIT
QKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVNLLKSKSKLG
VQVCLVFSIGQHIRDKNLMNSLITYLGCGYILKKNKSEFSWLEFCVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGL
DEIKKIKLNMNKGR

I-OnuI-e-CtxA (wildtype I-OnuI scaffold with 13 specificity-altering mutations from eOnuTherm-bCtxA)
SINPWILTGFADAEGSFGLRIRNRNKSSVGYSTELGFEIKLHNKDKSILENIQSTWKVGVIANSGSNAVRLRVTRFEDLKVIIDHFEKYPLIT
QKLGDYMLFKQAFCVMENKEHLKINGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVNLLKSKSKLG
VQVCLVFSIGQHIRDRNLMNSLITYLGCGYILEKNKSEFSWLEFCVTKFSDINDKIIPVFQENTLIGVKLEDFEDWCKVAKLIEEKKHLTESG
LDEIKKIKLNMNKGR

I-OnuI-Lib07 (last active intermediate prior to failed engineering towards the bCtxA target on the wildtype I-OnuI scaffold)
SINPWILTGFADAEGSFLLRIRNNNKSSVGYSTELGFQISLHNKDKSILENIQSTWKVGVIANRGDNAVSLKVTRFEDLKVIIDHFEKYPLIT
QKLGDYKLFKQAFSVMENKEHLKENGIKELVRIKAKLNWGLTDELKKAFPENISKERSLINKNIPNFKWLAGFTSGEGNFLVRLIKSKSKLG
VQVQLVFAIGQHIRDKNLMNSLITYLGCGYILEKNKSEFSWLEFVVTKFSDINDKIIPVFQENTLIGVKLEDFEDWCKVAKLIEEKKHLTESG
LDEIKKIKLNMNKGR

I-OnuI-e-Therm-hChr11v1 (eOnuTherm-hChr11v1) (Intermediate #1)
SINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHFEKYPLITQ
KYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGDGCFFVNLIKSKSKLGVQ
VQLVFSISQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLEFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDE
IKKIKLNMNKGR

I-OnuI-e-Therm-hChr11v2 (eOnuTherm-hChr11v2) (Intermediate #2)
SINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHFEKYPLITQ
KYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGDGCFFVNLSKKKTKLGV
QVKLVFSISQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLEFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLD
EIKKIKLNMNKGR

I-OnuI-e-Therm-hChr11v3 (eOnuTherm-hChr11v3) Final engineered enzyme
SINPWILTGFADAEGSFLLRIRKYSQTRVGYLTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHFEKYPLITQ
KYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGDGCFFVNLSKKKTKLGV
QVKLVFSISQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLEFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLD
EIKKIKLNMNKGR

Table S2: Sequences of Oligonucleotides in this Study

Listed in a 5'→3' orientation, all oligonucleotides ordered from Integrated DNA Technologies (IDT); **related to STAR Methods**.

Labeled Double-stranded Target Site Substrates

A647 Target Site Substrate Primer	5′ (5Alex647N) – TGGACACGACTTGAGC 3′
Biotin Target Site Substrate Primer	5′ (5Biosg) – TCAGCACAGCACTACG 3′
Sample Target Site Substrate Template	5′ TGGACACGACTTGAGC**TTTCCACTTATTCAACCTTTTA**CGTAGTGCTGTGCTGA 3′ (22bp meganuclease target site for I-OnuI shown in blue text)

Sample Ultramers for Generating Engineering Library Inserts

DNA-contacting positions are highlighted to indicate potential places for incorporation of degenerate codons

Wild type I-OnuI Ultramer A

GGTTTCGCTGACGCTGAGGGT**TCT**TTC**CTG**CTG**AGA**ATC**AGA**AAC**AAC**AAC**AAGTCTTCTGTCGGT**TAT**TCT**ACT**GAA**CTG**GGT**TTC**CAG**ATC**ACC**CTGCACAACAAGGACAAGTCCATCCTG

Wild type I-OnuI Ultramer B

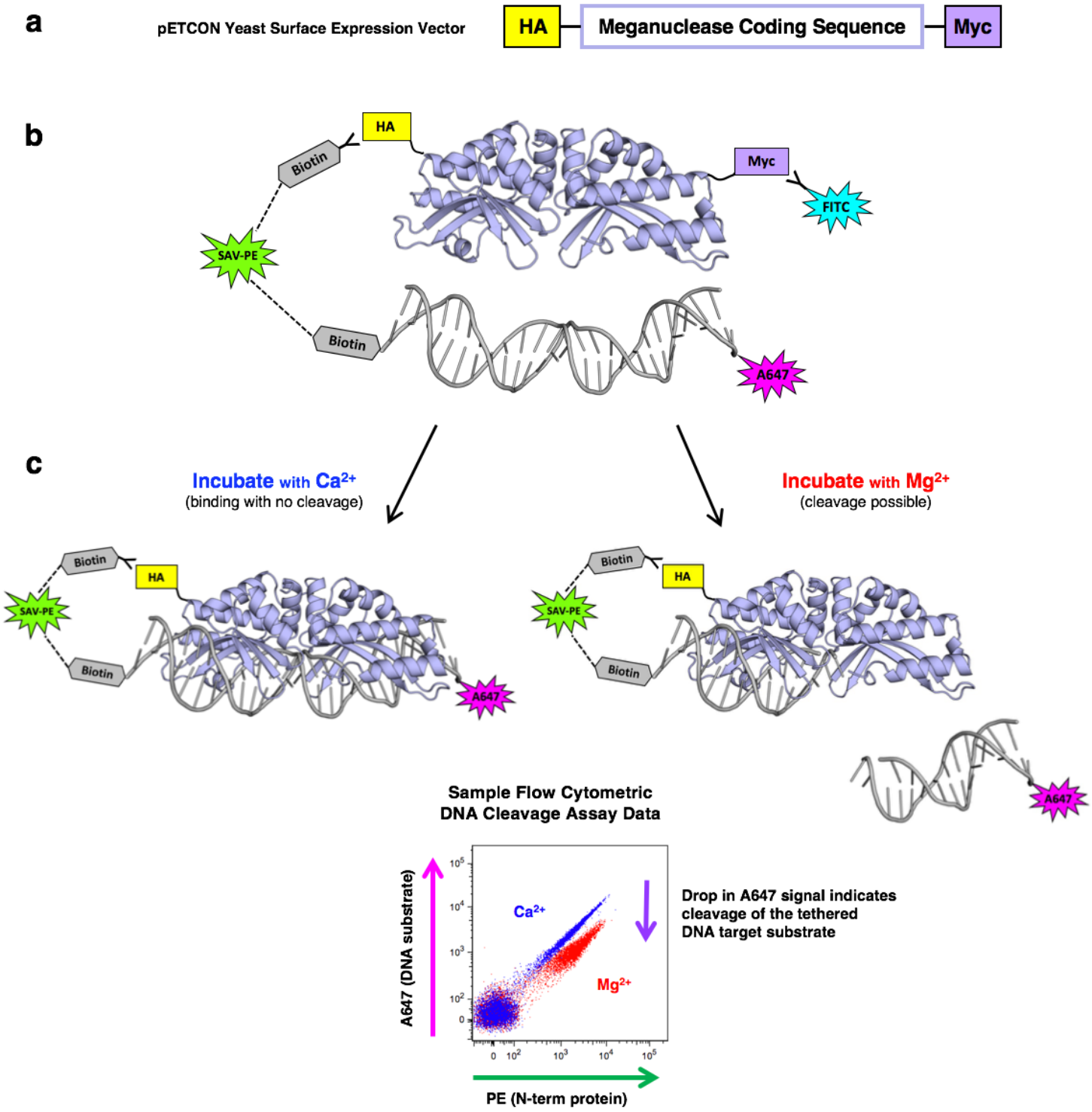
CATCCAGTCCACTTGGAAGGTCGGT**GTC**ATC**GCT**AAC**TCT**GGTGAC**AACGCT**GTC**TCT**CTG**AAG**GTC**ACC**AGATTCGAGGACCTGAAGGTCATCATCGAC

Wild type I-OnuI Ultramer C

GCTGGTTTCACTAGTGGTGAGGGT**TGT**TTC**TTC**GTC**AAC**CTG**ATC**AAG**TCCAAGTCCAAGCTGGGT**GTC**CAG**GTC**CAG**CTG**GTC**TTC**TCT**ATC**ACT**CAGCACATCAAGGACAAGAACCTGATGA

Wild type I-OnuI Ultramer D

CTCTGATCACTTATCTGGGTTGCGGT**TAT**ATC**AAG**GAG**AAG**AAC**AAG**TCCGAG**TTC**TCC**TGG**CTG**GAC**TTC**GTC**GTC**ACC**AAGTTCTCCGACATTAACGACAAGATCATCCCAGTCTTCCAGGAA



Supplementary Figure S1; related to STAR Methods. Schematic of the tethered flow cytometric DNA cleavage activity assay. (a) The pETCON vector expresses the meganuclease of interest on the surface of yeast with an N-terminal HA epitope tag and a C-terminal Myc epitope tag. (b) Three different fluorescent colors are utilized in this flow cytometric assay: 1.) FITC: The Myc tag is stained with an anti-Myc-FITC antibody to detect full-length expression of the protein. 2.) PE: The HA tag is stained with a biotinylated anti-HA antibody which is then conjugated to a fluorescent streptavidin (SAV-PE) molecule to detect the N-terminus of the protein. 3.) A647: The anti-HA-biotin-SAV-PE bridge can also be used to create a physical tethering of an A647-labeled double-stranded DNA substrate to the N-terminus of the protein. (c) Cleavage activity of the surface-expressed meganuclease is assessed by comparing A647 signal after incubation at 37°C in the presence of Ca^{2+} (blue cell population, enzyme can bind the DNA substrate but not cleave it) or Mg^{2+} (red cell population, binding and cleavage of the DNA substrate may occur). When the tethered DNA substrate is successfully cleaved, the A647 tag is released from the DNA and washed away. Therefore, a drop in the A647 signal indicates cleavage of the DNA substrate by the meganuclease. The cleavage shift can be quantitated by calculating a ratio of the median A647 signal for the Ca^{2+} sample to the median A647 signal for the Mg^{2+} sample.

a

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I-SmaMI         SKGENSKLNPWAVVGFIDAEGSFMVRVRKNSKYKTGWLVVAIFSVTVDKKDLFLLESLKTFFGGLGSIKKSGNSTFSYRI
eSmaGATA        SKGENSKLNPWAVVGFIDAEGSFMVRVRKRSDMKTGWLVTAIFSVGVDKKDLFLLESLKTFFGGLGSIKKAGNSVFLYRI
                ******************************.*. ******.***** ***********************:***.* ***

I-SmaMI         ESSEQLTKIILPFFDKYSLITEKLGDYLLFKKVLELMGTKEHLTQRGLEKIVSLKASINKGLSEELQAAFPQCVPTPRPE
eSmaGATA        YSSEQLTKIILPFFDKYSLITEKLGDYLLFKKVLELMGTKEHLTQRGLEKIVSLKASINKGLSEELQAAFPQCVPTPRPE
                 ********************************************************************************

I-SmaMI         INNKLIPDPFWLAGFVSGDGSFKSILKKSESIKVGFQSILVFQITQHARDVKLMESLISYLGCGFIEKDSRGPWLYYTVT
eSmaGATA        INNKNIPDPFWLAGFVSGDGSFKSYLTKAEWSKVGFQSHLEFQITQHARDVKLMESLISYLGCGYISKDSRGPWLYYRVT
                **** ****************** *.:*  ****** * **********************:*.********** **

I-SmaMI         NFSDIQGKIIPFFHQYKIIGSKYGDYMDWCKIALIMQNKNHLTPEGLNEIRALKGGMNKGRL
eSmaGATA        RFSDIQGKIIPFFHQYKIIGSKYGDYNDWCKIALIMQNKNHLTPEGLNEIRALKGGMNKGRL
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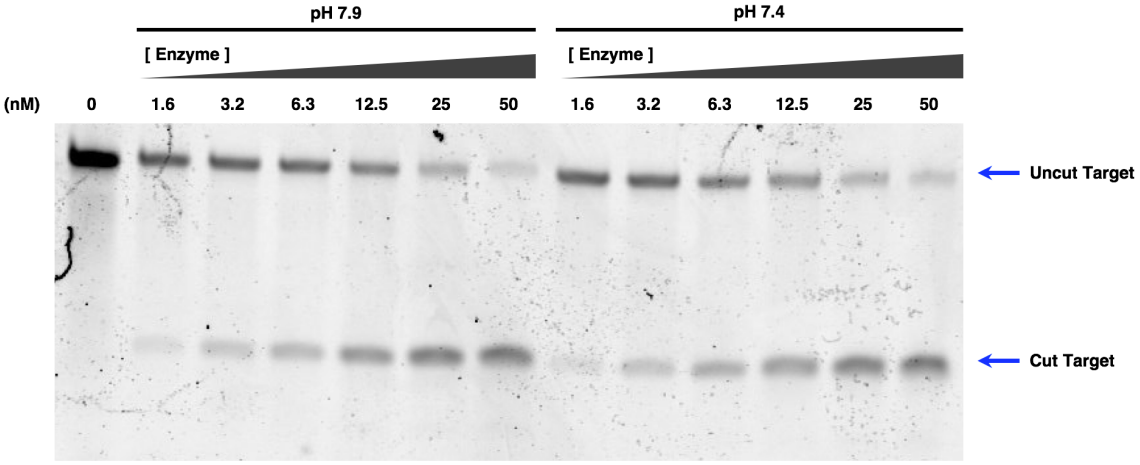
b



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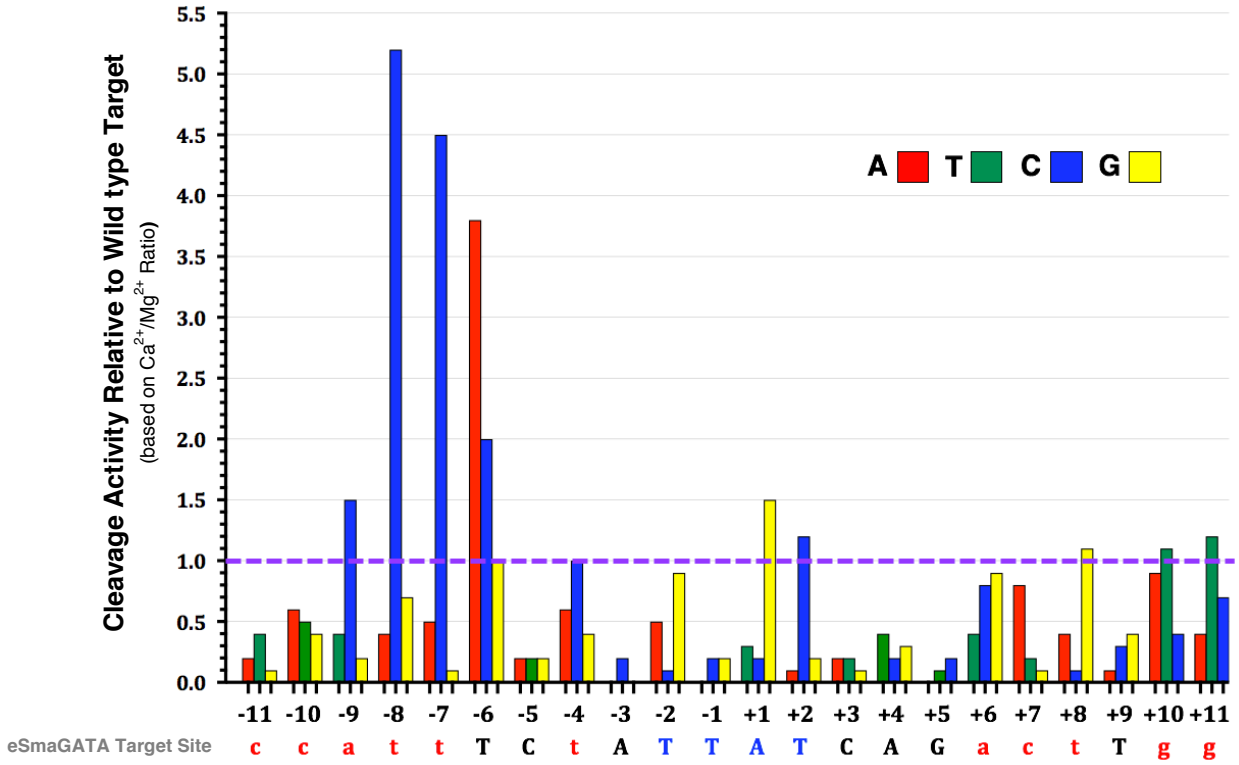

Supplementary Figure S2; related to Figure 1. Characterization of eSmaGATA (a) Amino acid sequence alignment of the initial engineering scaffold (I-SmaMI) and the final eSmaGATA meganuclease. (b) DNA cleavage activity of the eSmaGATA meganuclease. Recombinant eSmaGATA meganuclease was purified and tested for cleavage activity using fluorescently labeled double stranded DNA target substrates. Increasing concentration of enzyme was incubated with 40nM DNA target substrate for 20 minutes at 37°C. The assay was performed at both pH 7.9 and pH 7.4. The resulting cleavage products were separated on an acrylamide gel and visualized with a Typhoon imager. (c) DNA cleavage specificity of the final re-engineered eSmaGATA meganuclease. The ability of eSmaGATA to cleave 66 'miscognate' DNA target sites (each harboring a single base pair substitution at one of the 22 positions in the target sequence) was measured with the tethered flow cytometric cleavage assay. Activity against each target site is illustrated as a bar graph showing the Ca^{2+}/Mg^{2+} ratio (magnitude of the drop in A647 signal from the cleaved DNA target substrate) relative to activity against the cognate target site. Activity of the enzyme against the cognate target site is indicated with a horizontal purple line.

a

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wt      SRRESINPWILTGFADAEGSFLLRIRNNNKSSVGYSTELGFQITLHNKDKSILENIQSTWKVGVIANSGDNAVSLKVTRFEDLKVIIDHF
P1      SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWKVGVIANSGDNAVSLKVTRFEDLKVIIDHF
P3      SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWKVGVIANSGDNAVSLKVTRFEDLKVIIDHF
P4      SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHF
P5      SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHF
P6      SRRESINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHF
P7      SRRESINPWILTGFADAEGSFLLRIRKNSKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLKVIIDHF
        ****************************:*.******************************** ****************************

wt      EKYPLITQKLGDYMLFKQAFCVMENKEHLKINGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
P1      EKYPLITQKLGDYMLFKQAFCVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
P3      EKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
P4      EKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
P5      EKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKASLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
P6      EKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKASLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
P7      EKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKASLNWGLTDELKKAFPEIISKERSLINKNIPNFKWLAGFTSGEGCFFVN
        ********* .********* ********.*:**********.********************************************

wt      LIKSKSKLGVQVQLVFSITQHIKDKNLMNSLITYLGCGYIKEKNKSEFSWLDFVVTKFSDINDKIIPVFQENTLIGVKLEDFEDWCKVAK
P1      LIKSKSKLGVQVQLVFSITQHIKDKNLMNSLITYLGCGYIKEKNKSEFSWLDFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAK
P3      LIKSKSKLGVQVQLVFSITQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAK
P4      LIKSKSKLGVQVQLVFSITQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFEDWCKVAK
P5      LIKSKSKLGVQVQLVFSITQHIRDKNLMNSLIEYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDKIIPFFQEYTLIGTKLKDFLDWCKVAK
P6      LIKSKSKLGVQVQLVFSITQHIRDKNLMNSLIEYLGCGYIKKKNKSEFSWLDYVVTKFSDIRDKIIPFFQEYTLIGTKYKDFLDWCKVAK
P7      LIKSKSKLGVQVQLVFSITQHIRDKNLMNSLIEYLGCGYIKKKNKSSFSWLDYVVTKFSDIRDKIIPFFQKYTLIGTKYKDFLDWCKVAE
        **********************:********* ********:****.*****:********.*****.**: ****.* :** ******:

wt      LIEEKKHLTESGLDEIKKIKLNMNKGRVF
P1      LIEEKKHLTEEGLDEIKKIKLNMNKGRVF  9 mutations
P3      LIEEKKHLTEEGLDEIKKIKLNMNKGRVF  14 mutations
P4      LIEEKKHLTEEGLDEIKKIKLNMNKGRVF  15 mutations
P5      LIENKKHLTEEGLDEIKKIKLNMNKGRVF  19 mutations
P6      LIENKKHLTEEGLDKIKKIKLNMNKGRVF  22 mutations
P7      LIENKKHLTEEGLDKIKKIKLNMNKGRVF  26 mutations
        ***:******.***:***************
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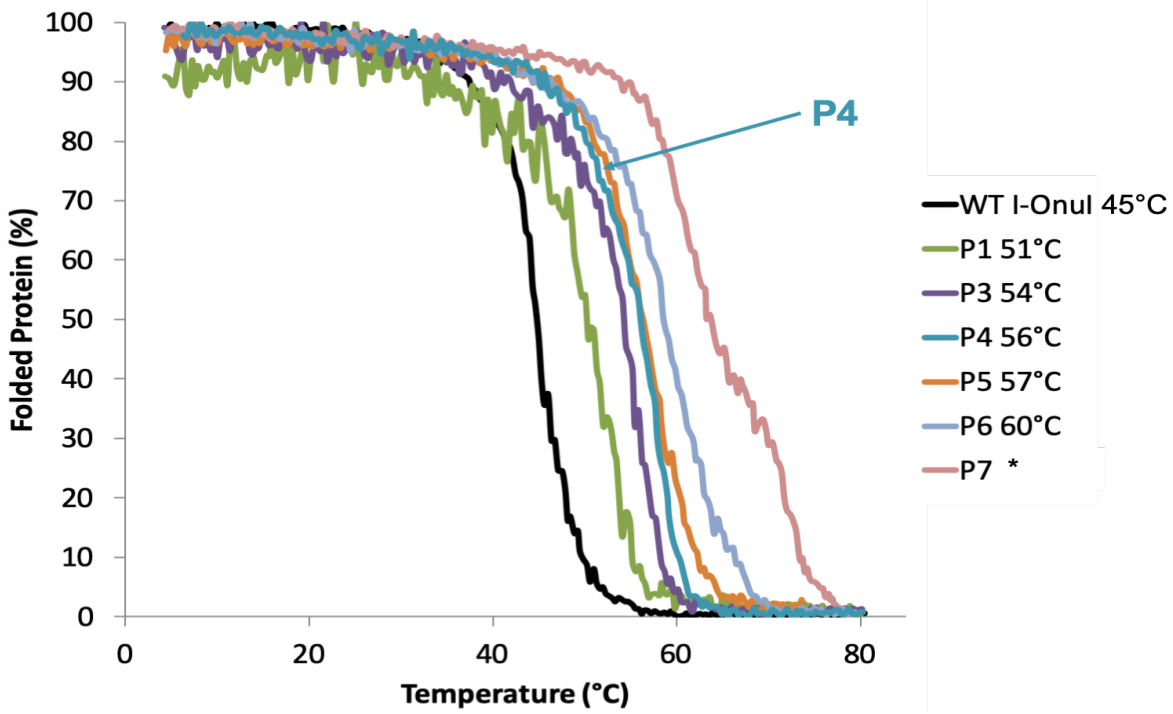
b



Supplementary Figure S3; related to Figure 2. Structure-based design variants of the I-OnuI meganuclease tested for improved thermal stability. (a) A series of 6 constructs with increasing numbers of suggested point mutations (generated by the PROSS server) were synthesized and individually expressed and purified. (P2 was removed because it was redundant with P1.) (b) Examination of the thermal stability of each construct using thermal denaturation analyses via circular dichroism (CD) spectroscopy indicated thermal denaturation midpoints ranging from 45° C (the wild-type enzyme) to 60°C or higher ('P6', containing 22 point mutations and 'P7', containing 26 point mutations; the P7 construct displays a somewhat biphasic denaturation transition that makes the estimation of the Tm value less accurate). In addition to the extent of Tm increases displayed by the various constructs, the overall cooperativity of protein unfolding varied slightly. We eventually selected construct 'P4' (harboring 15 amino acid substitutions and displaying highly cooperative unfolding behavior with a Tm value of 56°) for use as a thermostabilized scaffold for engineering and for comparative analyses vs. engineering with the wild-type I-OnuI scaffold. 'P4' is eOnuTherm in this manuscript.

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eOnuTherm             SINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLK
eOnuTherm-bCtxA       SINPWILTGFADAEGSFGLRIRKRNKSSVGYSTELGFEIKLHNKDKSILENIQSTWGVGVIANSGSNAVRLRVTRFEDLK
                      ***************** *****.*************:*.************************.*** *:********

eOnuTherm             VIIDHFEKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNF
eOnuTherm-bCtxA       VIIDHFEKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNF
                      ********************************************************************************

eOnuTherm             KWLAGFTSGEGCFFVNLIKSKSKLGVQVQLVFSITQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDK
eOnuTherm-bCtxA       KWLAGFTSGEGCFFVNLLKSKSKLGVQVCLVFSIGQHIRDKNLMNSLITYLGCGYILKKNKSEFSWLEFCVTKFSDIRDK
                      *****************:********** ***** ******************** **********:* **********

eOnuTherm             IIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDEIKKIKLNMNKGR
eOnuTherm-bCtxA       IIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDEIKKIKLNMNKGR
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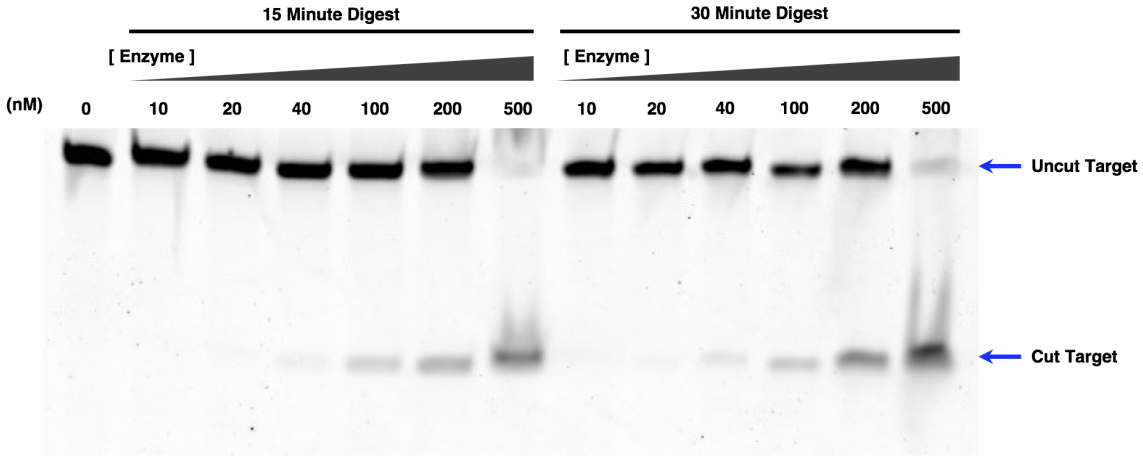

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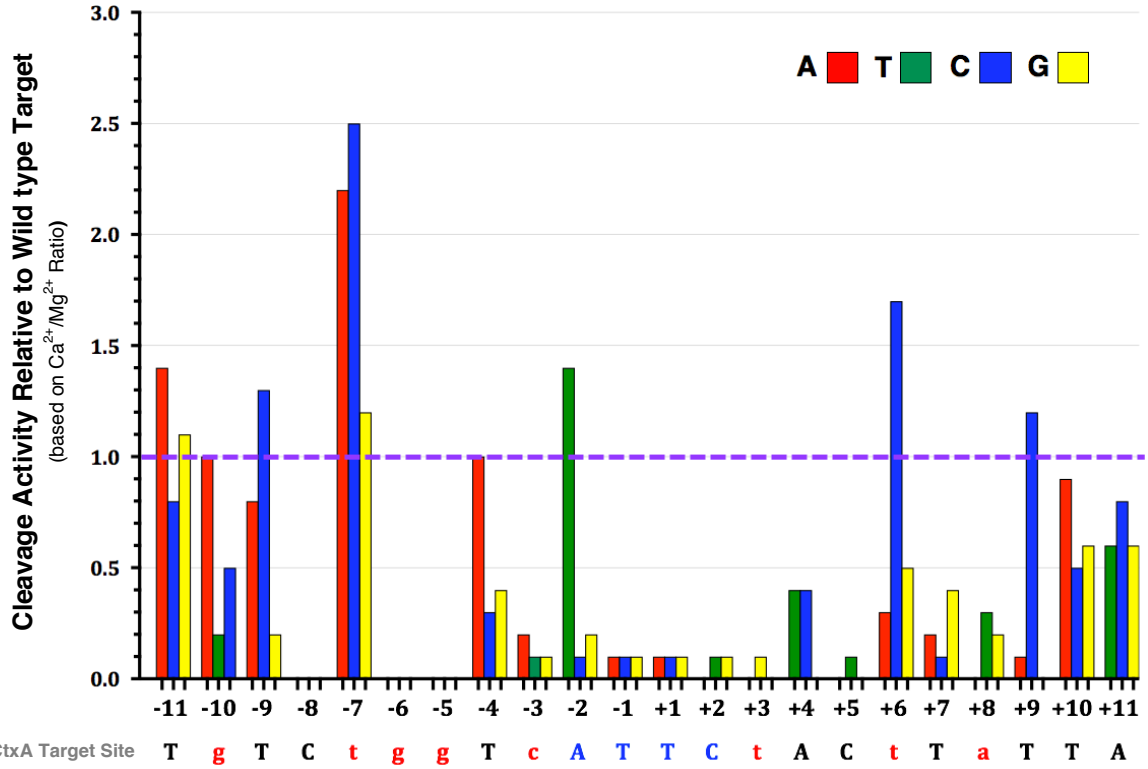
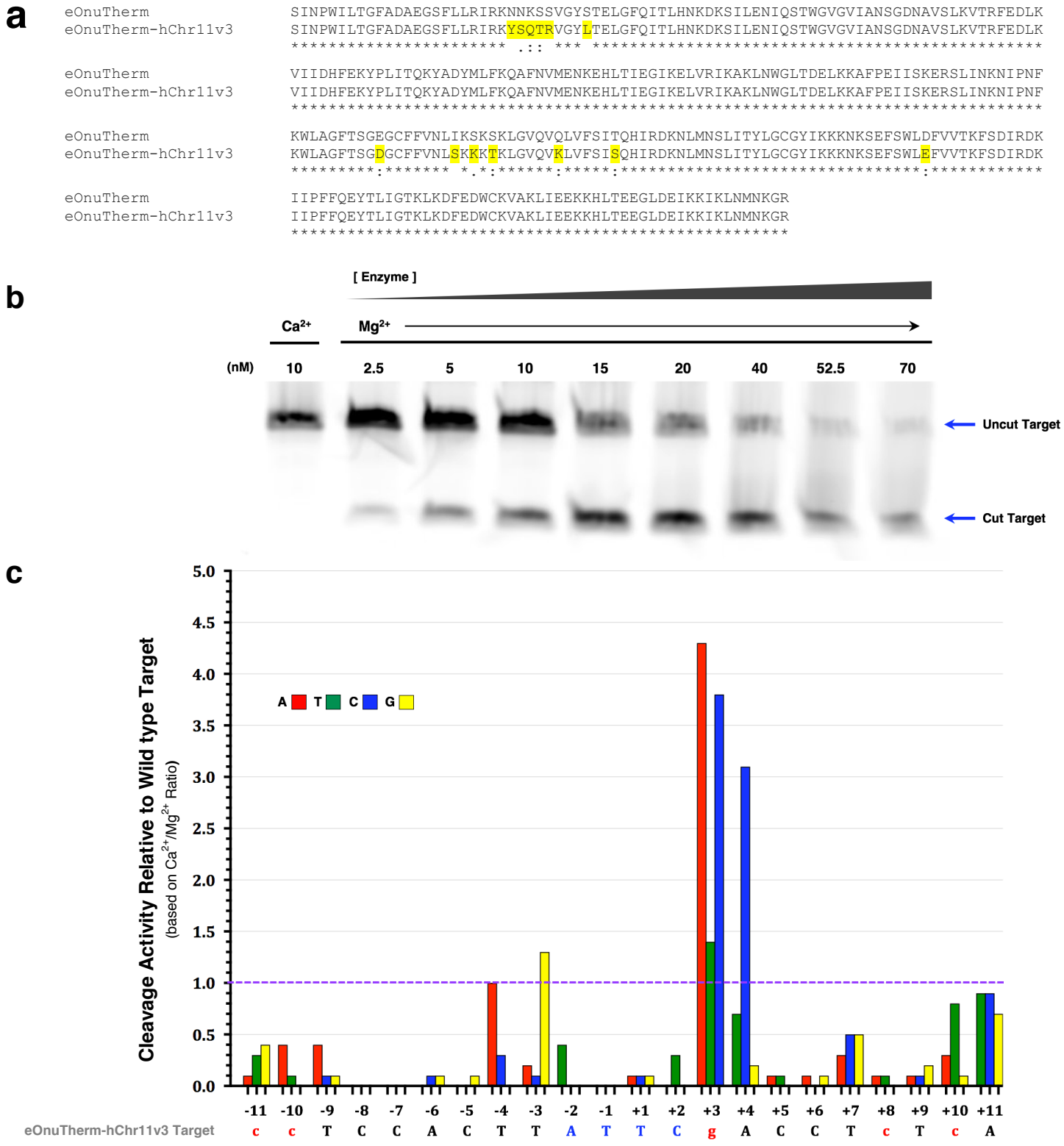


Supplementary Figure S4; related to Figure 3. Characterization of eOnuTherm-bCtxA (a) Amino acid sequence alignment of the initial engineering scaffold (eOnuTherm) and the final engineered eOnuTherm-bCtxA meganuclease. (b) DNA cleavage activity of the eOnuTherm-bCtxA meganuclease. Recombinant eOnuTherm-bCtxA meganuclease was purified and tested for cleavage activity using fluorescently labeled double-stranded DNA target substrates. Increasing concentration of enzyme was incubated with 40nM DNA target substrate at pH 7.9 and a temperature of 37°C. The digest was allowed to run for 15 minutes or 30 minutes. The resulting cleavage products were separated on an acrylamide gel and visualized using a Typhoon imager. (c) DNA cleavage specificity of the final re-engineered eOnuTherm-bCtxA meganuclease. The ability of eOnuTherm-bCtxA to cleave 66 'miscognate' DNA target sites (each harboring a single base pair substitution at one of the 22 positions in the target sequence) was measured with the tethered flow cytometric cleavage assay. Activity against each target site is illustrated as a bar graph showing the Ca^{2+}/Mg^{2+} ratio (magnitude of the drop in A647 signal from the cleaved DNA target substrate) relative to activity against the cognate target site. Activity of the enzyme against the cognate target site is indicated with a horizontal purple line.



Supplementary Figure S5; related to Figure 4. Characterization of eOnuTherm-hChr11v3

(a) Amino acid sequence alignment of the initial engineering scaffold (eOnuTherm) and the final engineered eOnuTherm-hChr11v3 meganuclease. (b) DNA cleavage activity of the eOnuTherm-hChr11v3 meganuclease. Recombinant eOnuTherm-hChr11v3 meganuclease was purified and tested for cleavage activity using fluorescently labeled double-stranded DNA target substrates. Increasing concentration of enzyme was incubated with 40nM DNA target substrate at pH 7.4 and a temperature of 37°C for 30 minutes. A sample with 10nM enzyme and Ca^{2+} as the divalent cation was used for a no-cleavage control, while the remaining reactions were performed in the presence of Mg^{2+}. The resulting cleavage products were separated on an acrylamide gel and visualized with a Typhoon imager. (c) DNA cleavage specificity of the final engineered eOnuTherm-hChr11v3 meganuclease. The ability of eOnuTherm-hChr11v3 meganuclease to cleave 66 'miscognate' DNA target sites (each harboring a single base pair substitution at one of the 22 positions in the target sequence) was measured with the tethered flow cytometric cleavage assay. Activity against each target site is illustrated as a bar graph showing the Ca^{2+}/Mg^{2+} ratio (magnitude of the drop in A647 signal from the cleaved DNA target substrate) relative to activity against the cognate target site. Activity of the enzyme against the cognate target site is indicated with a horizontal purple line.

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eOnuTherm       SINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLK
Intrm_1         SINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLK
Intrm_2         SINPWILTGFADAEGSFLLRIRKNNKSSVGYSTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLK
Final           SINPWILTGFADAEGSFLLRIRKYSQTRVGYLTELGFQITLHNKDKSILENIQSTWGVGVIANSGDNAVSLKVTRFEDLK
                ********************** .:: *** ********************************************

eOnuTherm       VIIDHFEKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNF
Intrm_1         VIIDHFEKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNF
Intrm_2         VIIDHFEKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNF
Final           VIIDHFEKYPLITQKYADYMLFKQAFNVMENKEHLTIEGIKELVRIKAKLNWGLTDELKKAFPEIISKERSLINKNIPNF
                ********************************************************************************

                        *
eOnuTherm       KWLAGFTSGEGCFFVNLIKSKSKLGVQVQLVFSITQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLDFVVTKFSDIRDK
Intrm_1         KWLAGFTSGDGCFFVNLIKSKSKLGVQVQLVFSISQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLEFVVTKFSDIRDK
Intrm_2         KWLAGFTSGDGCFFVNLSKKKTKLGVQVKLVFSISQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLEFVVTKFSDIRDK
Final           KWLAGFTSGDGCFFVNLSKKKTKLGVQVKLVFSISQHIRDKNLMNSLITYLGCGYIKKKNKSEFSWLEFVVTKFSDIRDK
                *********:******* *.*:******:*****:*********************************:************

eOnuTherm       IIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDEIKKIKLNMNKGRVF
Intrm_1         IIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDEIKKIKLNMNKGRVF  3 mutations
Intrm_2         IIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDEIKKIKLNMNKGRVF  7 mutations
Final           IIPFFQEYTLIGTKLKDFEDWCKVAKLIEEKKHLTEEGLDEIKKIKLNMNKGRVF  13 mutations
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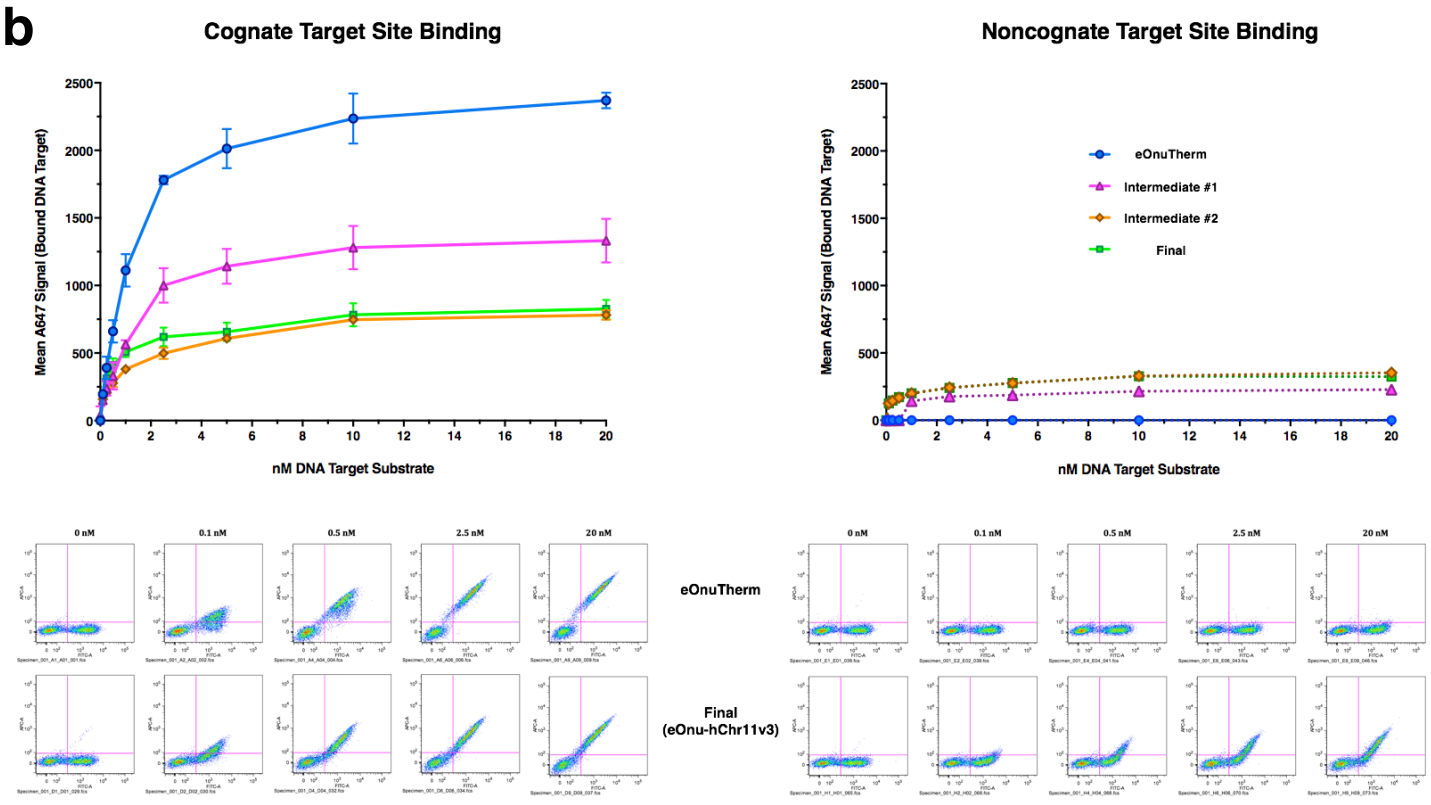
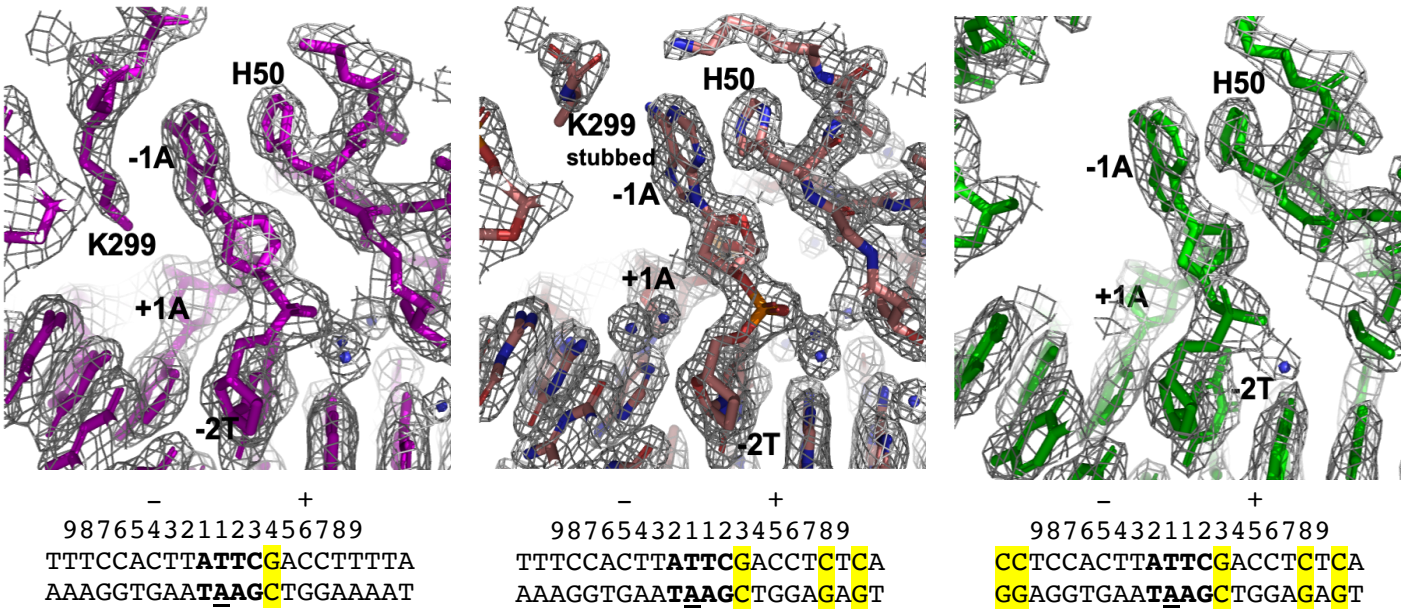
b



Supplementary Figure S6; related to Figures 4 and 5. Sequence alignment and DNA binding by the eOnuTherm-hChr11 enzyme series. (a) Amino acid sequence alignment of the initial engineering scaffold (eOnuTherm), two intermediates along the engineering pathway, and the final eOnuTherm-hChr11v3 meganuclease. The active site glutamic acid altered in the E178D mutant is designated by a red asterisk. **(b) Cognate (specific) and noncognate (nonspecific) DNA binding by a series of engineered enzymes.** Each of the four enzymes along the eOnuTherm-hChr11 engineering pathway were expressed on the yeast surface and incubated with increasing concentrations of labeled DNA target substrates. Binding was assayed against the cognate target site for each enzyme (see **Figures 5** and **S7** for target site sequences) as well as a noncognate DNA target (ACTTCAGATGTAGACTGTCAGT). ***Top***: Binding of the various DNA target substrates over a 0nM-20nM range of concentrations was quantified via flow cytometric analysis. Three replicates of the experiment were collected from separately induced yeast cultures. Error bars represent standard deviation from the mean. ***Bottom***: Sample flow cytometric plots are shown for a select number of DNA concentrations (0nM, 0.1nM, 0.5nM, 2.5nM, and 20nM). Spots in the upper right quadrant represent yeast cells with full-length surface expressed meganuclease (anti-Myc-FITC signal) binding the A647-labeled DNA target substrates.



WT I-OnuI
TTTCCACTTATTCAACCTTTTA
AAAGGTGAATAAGTTGGAAAAT

Supplementary Figure S7; related to Figure 6. Flipping of Adenine -1. This structural rearrangement (the largest in the protein-DNA complex relative to the starting scaffold and its bound DNA target) appears to be associated with the initial incorporation of the E178D mutation in the enzyme active site near the center of the DNA target. The flipped base is underlined in each target sequence, and those basepairs that are altered from the wild-type target sequence are highlighted in yellow. The electron density is displayed at a 1σ contour level.