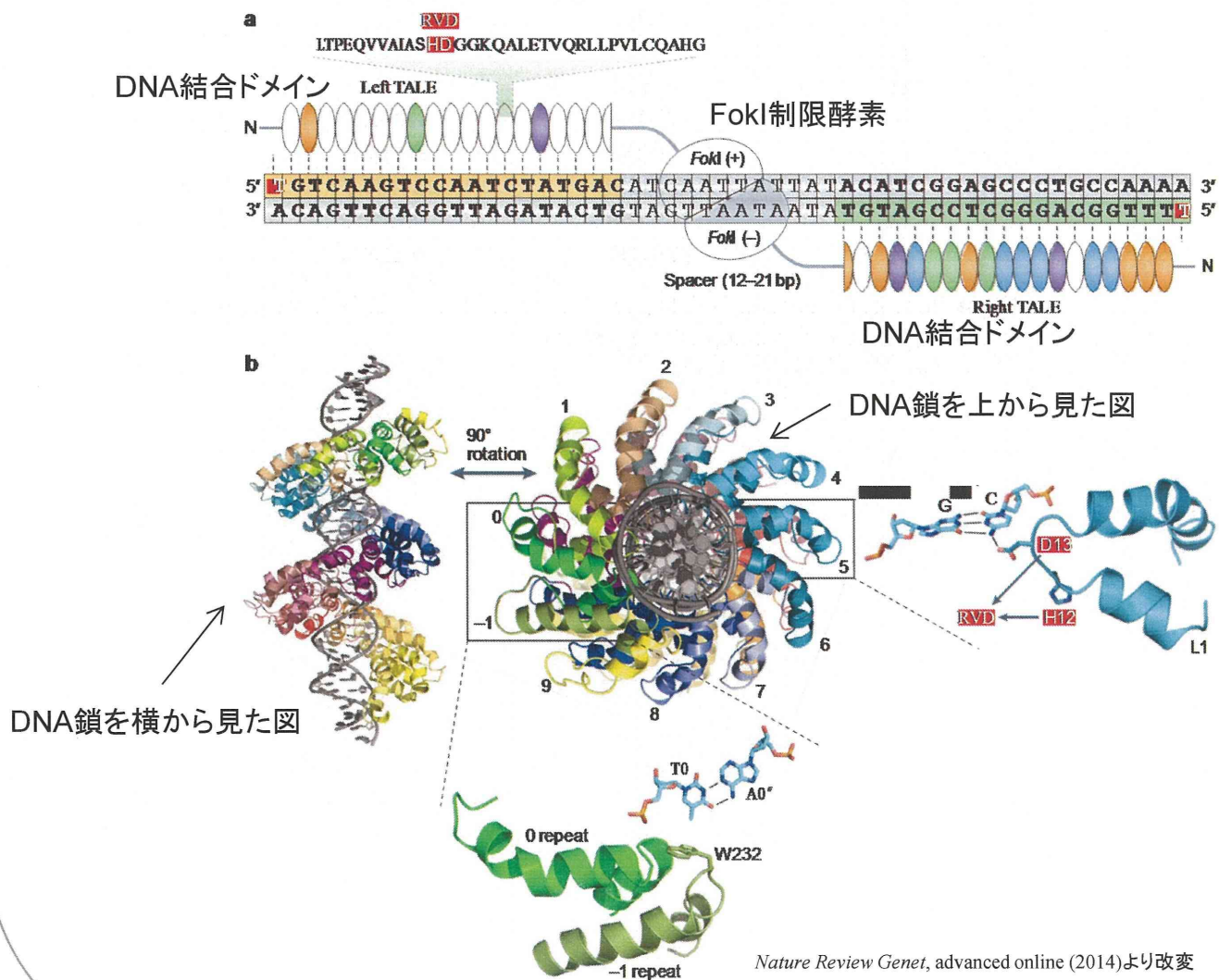


Trends in Biotechnology 31, 397 (2013)より (right panel)



Nature Review Genet, advanced online (2014)より改変

Fig.3 Structure of TALEN

Mutations in site	Off-target sites to CCR5A	Statistically expected
0	1	1
1	0	0.0
2	0	0.0
3	0	0.0
4	0	0.0
5	0	0.0
6	0	0.0
7	0	0.3
8	8	3.6
9	7	34.1
	0	
10	634	275.9
11	4338	1956.3
12	27114	12226.7
13	149005	67716.9
14	648230	333747.3
15	2657598	1468488.3
16	9783617	5782172.6

Fig.4 Potential genomic off-target sites related to *CCR5A* on-target site
(片側18bp、両側36bp TALEN使用)
Nature Methods, 11, 429 (2014)より

Cellular modification induced by TALENs at on-target and predicted off-target genomic sites

site	noTAIen (%)	CCR5A eI/KK	CCR5A eId/KKr	CCR5A	number of mutation
		Foki (%)	Foki (%)	homo Foki (%)	
OnCCR5A	<0.006	9.8	28	47	0 (on-target)
OffC-5	<0.006	0.53	2.3	2.3	11
OffC-15	<0.020	<0.014	0.23	0.043	
OffC-16	<0.006	<0.006	0.031	<0.006	
OffC-28	<0.009	0.014	0.16	0.056	
OffC-36	<0.006	<0.006	0.15	0.028	
OffC-38	<0.006	ND	ND	0.067	
OffC-49	<0.006	ND	ND	0.110	
OffC-69	<0.010	ND	ND	0.089	
OffC-76	<0.006	ND	ND	0.149	9

site	noTAIen (%)	ATM eI/KK	ATM eId/KKr	ATM	number of mutation
		Foki (%)	Foki (%)	homo Foki (%)	
OnATM	0.007	6.8	16	18	0 (on-target)
OffA-1	<0.006	<0.006	0.026	0.077	
OffA-11	<0.006	<0.006	0.036	0.39	10
OffA-13	<0.006	0.008	0.025	<0.006	
OffA-16	<0.006	<0.006	<0.006	0.057	
OffA-17	<0.051	<0.14	<0.17	0.94	9
OffA-23	0.018	<0.006	0.29	0.23	
OffA-35	<0.006	<0.006	<0.006	0.070	

Fig.5A Cellular modification rate (%) at on-target and off-target genomic sites

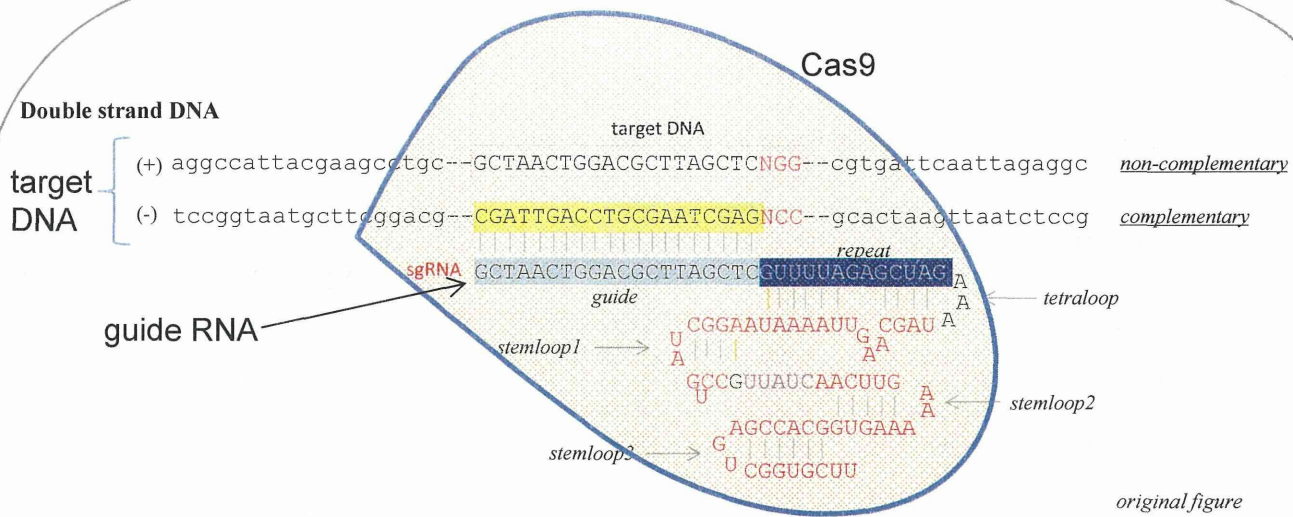
Nature Methods, 11, 429 (2014)より改変

CCR5A Site	Score	Mut.	Left half-site	Spacer length	Right half-site
OnCCR5A	0.008	0	TTCATTACACCTGCAGCT	18	AGTATCAATTCTGGAAGA
OffC-1	0.747	9	TaCATcACAtaTGCAaaT	29	tGTATCAtTTCTGGgAGA
OffC-2	0.747	9	TaCATcACAtaTGCAaaT	29	tGTATCAtTTCTGGgAGA
OffC-3	0.747	9	TaCATcACAtaTGCAaaT	29	tGTATCAtTTCTGGgAGA
OffC-4	0.747	11	TcCATaACACaTcttttCT	10	tGcATCAtTcCTGGAAGA
★ OffC-5	0.804	11	TcCAaTACctCTGCcaCa	14	AGgAgCAAcTCTGGgAGA
OffC-6	0.818	10	TTCaGTcCATCTGaAaac	16	gGTATCAtTTCTGGAgGA
OffC-7	0.834	14	TaCAaaACcCtTGcCaaa	27	taTATCAATTtgGGgAGA
OffC-8	0.837	12	TcCAagACACCTGcTtac	26	tcTATCAATTtgGGgAGA
OffC-9	0.874	10	TTCATaACAtCTtaAaaT	27	AaTAcCAAcTCTGGAtGA
OffC-10	0.89	12	TcCAaaACAtCTGaAaaT	25	tGgATCAaaTtgGGAAGA

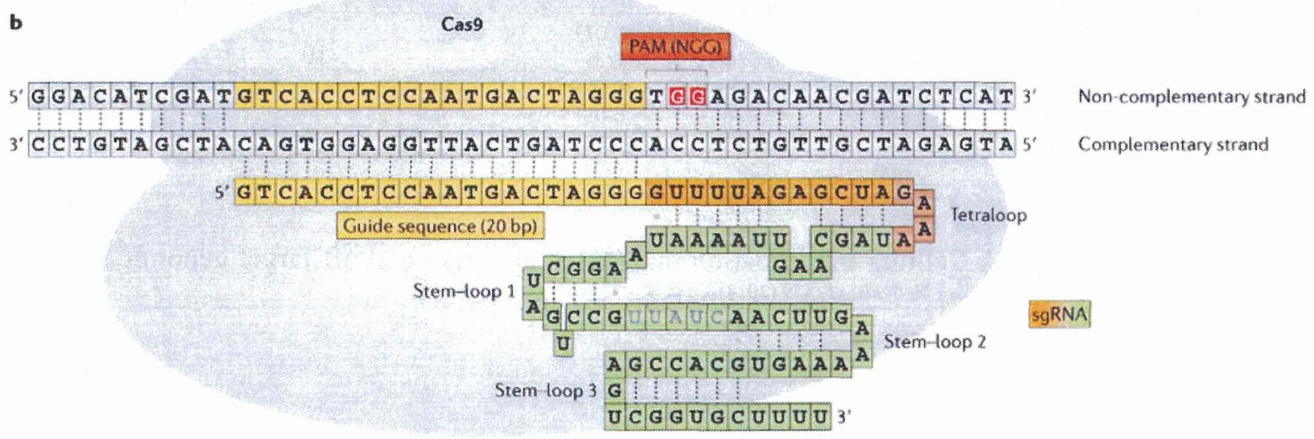
Fig.5B Predicted off-target sites

(2.3%で変異導入が検出されたon-targetサイトOffC-5でのミスマッチ塩基(小文字))

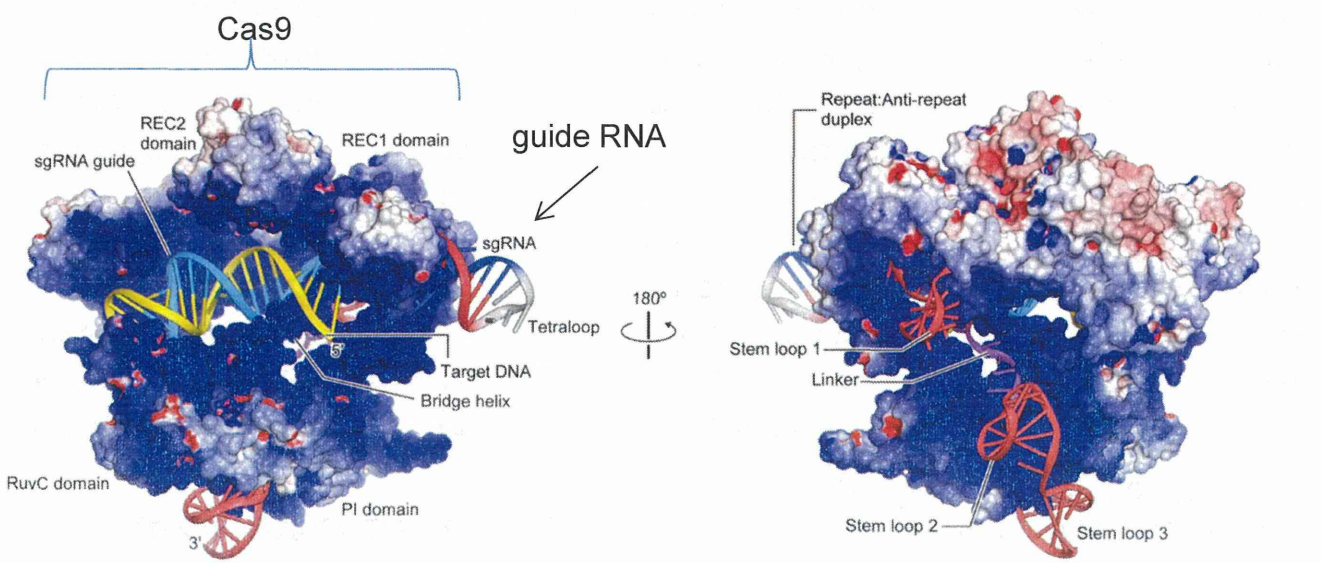
Nature Methods, 11, 429 (2014)より改変



original figure



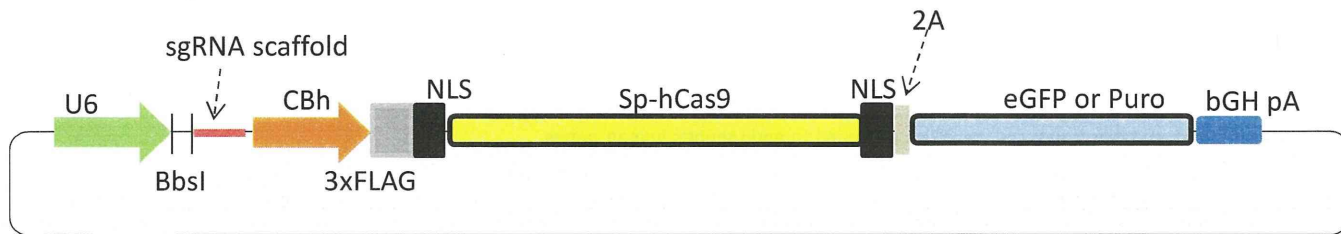
Nature Review Genet, advanced online (2014)より改変



Nishimatsu et al, Cell, 156, 935 (2014)より

Fig.6 Structure of CRISPR/Cas9 system

for mammalian expression



for plant expression

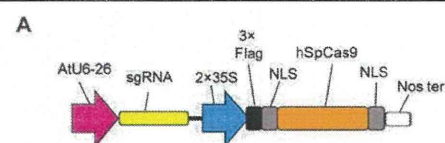
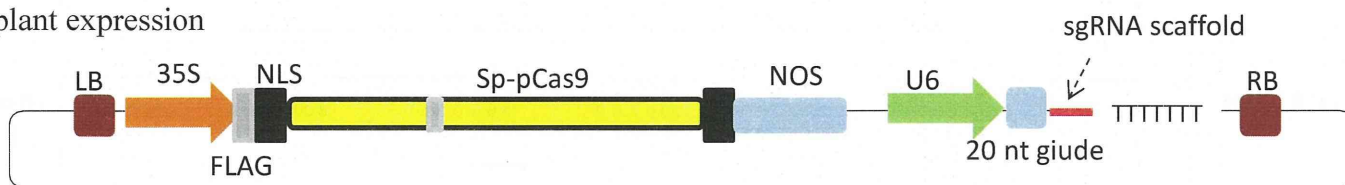
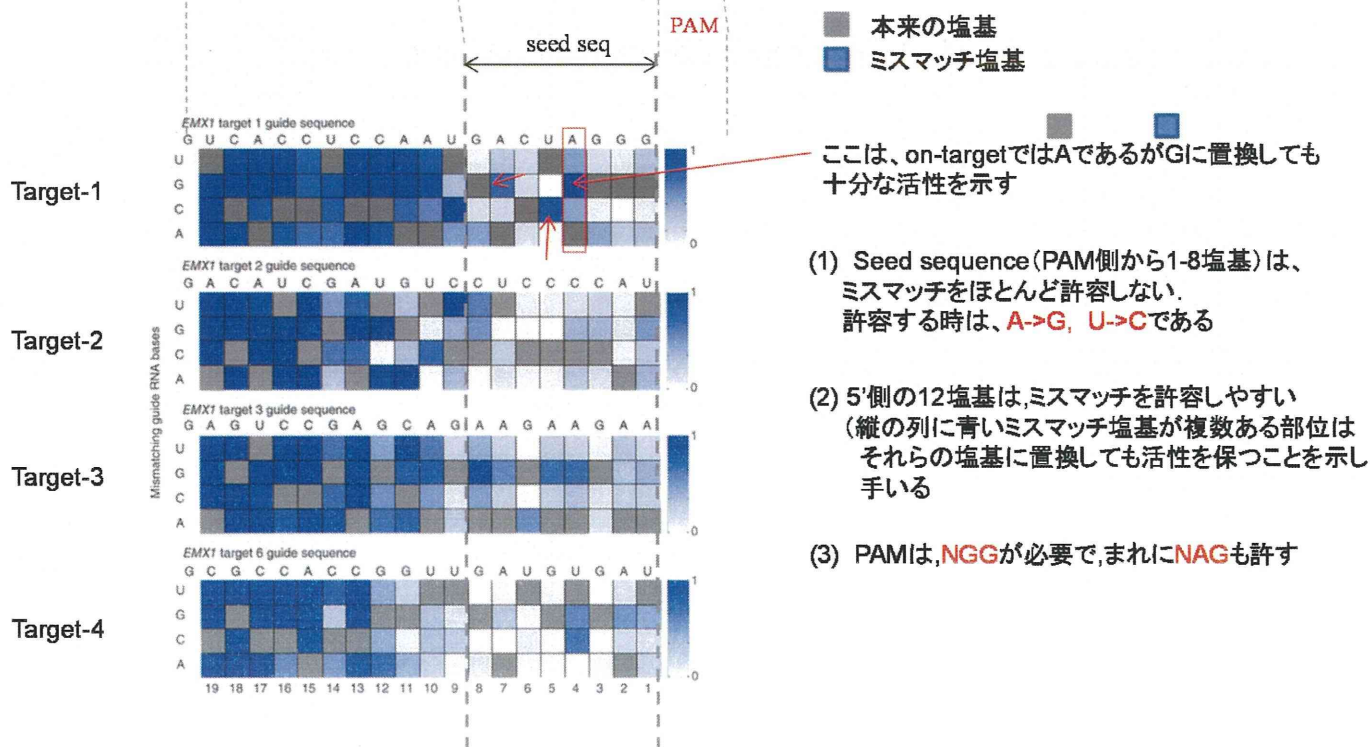


Fig.7 CRISPR/Cas plasmids for mammalian and plant

1塩基ずつmismatchを入れて、活性を比較

5' --GCTAACTGGACGCTTAGCTC NGG--3'



Hsu et al, Nat Biotech, 31, 827 (2013)より改変

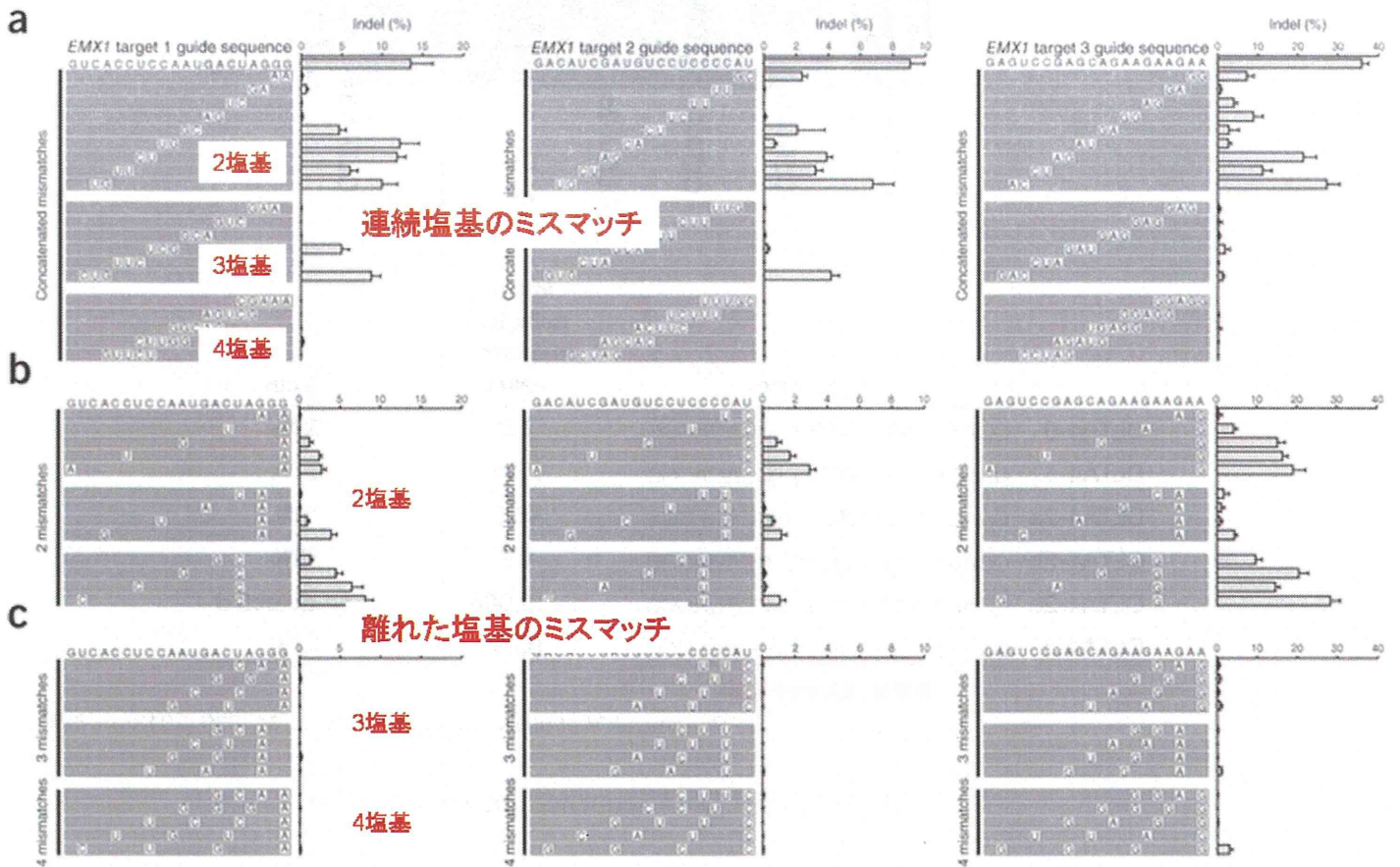
Fig.8 Single-nucleotide specificity of Cas9

On- and off-target mutations induced by RGNs designed to endogenous human genes

Target	Site name	Sequence	Indel mutation frequency (%) ± s.e.m.			Gene			
			U2OS.EGFP	HEK293	K562				
Target 1 (VEGFA site 1)	T1	GGGTGGGGGAGTTTGCTCCTGG	26.0 ± 2.9	10.5 ± 0.07	3.33 ± 0.42	VEGFA	on-target		
GC含量70%	OT1-3	GGATGGAGGGAGTTTGCTCCTGG	25.7 ± 9.1	18.9 ± 0.77	2.93 ± 0.04	IGDCC3	off-target		
	OT1-4	GGGAGGGTGGAGTTTGCTCCTGG	9.2 ± 0.8	8.32 ± 0.51	N.D.	LOC116437			
	OT1-6	CGGGGGAGGGAGTTTGCTCCTGG	5.3 ± 0.2	3.67 ± 0.09	N.D.	CACNA2D			
	OT1-11	GGGAGGGGAGTTTGCTCCTGG	17.1 ± 4.7	8.54 ± 0.16	N.D.				
Target 2 (VEGFA site 2)	T2	GACCCCTCCACCCCGCTCCGG	50.2 ± 4.9	38.6 ± 1.92	15.0 ± 0.25	VEGFA	on-target		
GC含量80%	OT2-1	GACCCCTCCACCCCGCTCCGG	14.4 ± 3.4	33.6 ± 1.17	4.10 ± 0.05	FMN1	off-target		
	OT2-2	GGGCTCCACCCCGCTCTGG	20.0 ± 6.2	15.6 ± 0.30	3.00 ± 0.06	PAX6			
	OT2-6	CTACCCCTCCACCCCGCTCCGG	8.2 ± 1.4	15.0 ± 0.64	5.24 ± 0.22	PAPD7			
	OT2-9	GCCCCACCCACCCCGCTCTGG	50.7 ± 5.6	30.7 ± 1.44	7.05 ± 0.48	LAMA3			
	OT2-15	TACCCCTCCACCCCGCTCTGG	9.7 ± 4.5	6.97 ± 0.10	1.34 ± 0.15	SPNS3			
	OT2-17	ACACCCCTCCACCCCGCTCAGG	14.0 ± 2.8	12.3 ± 0.45	1.80 ± 0.03				
	OT2-19	ATTCCCCCACCACCCCGCTCAGG	17.0 ± 3.3	19.4 ± 1.35	N.D.	HDLBP			
	OT2-20	CCCCACCCCTCCACCCCGCTCAGG	6.1 ± 1.3	N.D.	N.D.	ABLIM1			
	OT2-23	CGCCCTCCACCCCGCTCCGG	44.4 ± 6.7	28.7 ± 1.15	4.18 ± 0.37	CALY			
	OT2-24	CTCCCCACCCACCCCGCTCAGG	62.8 ± 5.0	29.8 ± 1.08	21.1 ± 1.68				
	OT2-29	TGCCCCCACCACCCCGCTCTGG	13.8 ± 5.2	N.D.	N.D.	ACLY			
	OT2-34	AGGCCCTCCACCCCGCTCAGG	2.8 ± 1.5	N.D.	N.D.				
	Target 3 (VEGFA site 3)	T3	GGTGAGTGAGTGTGTGCGTGTGG	49.4 ± 3.8	35.7 ± 1.26	27.9 ± 0.52		VEGFA	on-target
	GC含量60%	OT3-1	GGTGAGTGAGTGTGTGTGAGG	7.4 ± 3.4	8.97 ± 0.80	N.D.		(abParts)	off-target
OT3-2		AGTGAGTGAGTGTGTGTGGGG	24.3 ± 9.2	23.9 ± 0.08	8.9 ± 0.16	MAX			
OT3-4		GCTGAGTGAGTGTATGCGTGTGG	20.9 ± 11.8	11.2 ± 0.23	N.D.				
OT3-9		GGTGAGTGAGTGCGTGCGGTGG	3.2 ± 0.3	2.34 ± 0.21	N.D.	TPCN2			
OT3-17		GTTGAGTGAATGTGTGCGTGAGG	2.9 ± 0.2	1.27 ± 0.02	N.D.	SLIT1			
OT3-18		TGTGGTGAGTGTGTGCGTGAGG	13.4 ± 4.2	12.1 ± 0.24	2.42 ± 0.07	COMDA			
OT3-20		AGAGAGTGAGTGTGTGATGAGG	16.7 ± 3.5	7.64 ± 0.05	1.18 ± 0.01				

Fu et al, Nature Biotechnology, 31, 822 (2013)より改変

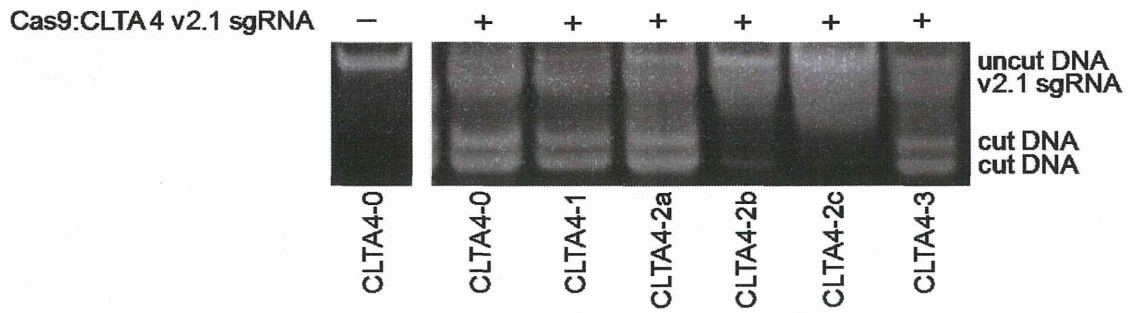
Fig.9 Single-nucleotide specificity of Cas9



ポイント: 3塩基以上のミスマッチはほとんど起きないため、2塩基ミスマッチ以内がゲノム上に存在しないように設計すればよい (GC含量が高く75%以上とか、PAM distal領域に集中した時には3-4塩基ミスマッチもあるが、設計で回避可能)

Hsu et al, Nature Biotechnology, 31, 827 (2013)より改変

Fig.10 Single-nucleotide specificity of Cas9

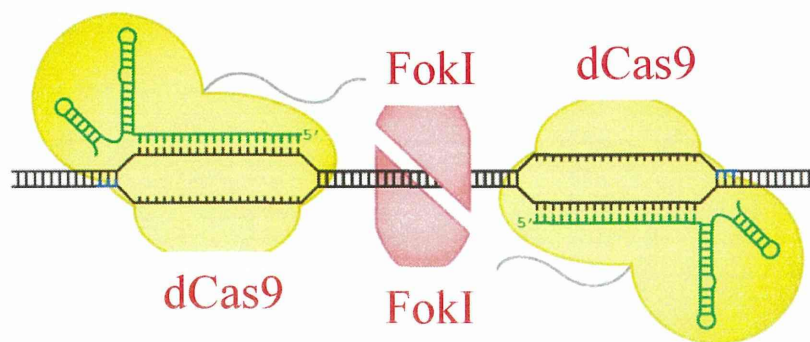


	sequence	<i>In vitro</i> selection enrichment value	% cut
CLTA4-0	GCAGATGTAGTGT T TCCACAGGG	7.9	85%
CLTA4-1	G a AGATGTAGTGT T TCCACAGGG	27.5	84%
CLTA4-2a	G a AGATGTAGTGT T TCCAC t GGG	43.9	79%
CLTA4-2b	GCAGAT Gg AG g GT T TCCACAGGG	1.0	35%
CLTA4-2c	GCAGATGTAGTGT Ta CCA g AGGG	0.064	none detected
CLTA4-3	Ggg GATGTAGTGT T TCCAC t GGG	95.9	72%

赤字は、ミスマッチ塩基

Pattanayak V *et al*, *Nature Biotechnology*, 31, 839 (2013)より改変

Fig.11 Off-target DNA sequence and cut ratio (%)



Tsai SQ, *et al*, *Nature Biotechnology*, doi:10.1038/nbt.2908 (2014)より改変

Fig.12 Off-target DNA sequence and cut ratio (%)

Deletions:

```

CTTGATGCCGTTCTTCTGGTCATCCTCATCCTGATAAACTGCAAAGAAGTGTTCGGCCATGATA wt
CTTGATGCCGTTCTTCTGGTCATCCTCATCCTGA-AACTGCAAAGAAGTGTTCGGCCATGATA -1
CTTGATGCCGTTCTTCTGGTCATCCTCATC-----TAAACTGCAAAGAAGTGTTCGGCCATGATA -4
CTTGATGCCGTTCTTCTGGTCATCCTCATCCTGA-----CAAAGAAGTGTTCGGCCATGATA -7
CTTGATGCCGTTCTTCTGGTCATCCTC-----ACTGCAAAGAAGTGTTCGGCCATGATA -10
CTTGATGCCGTTCTTCTGGT-----CTGATAAACTGCAAAGAAGTGTTCGGCCATGATA -10
CTTGATGCCGTTCTTCTGGTCATCCTCATCCTGA-----AACTGTTCGGCCATGATA -13
CTTGATGCCGTTCTTCTGGTCATCC-----AAGAAGTGTTCGGCCATGATA -19
CTTGATGCCGTTCTTCTGGTC-----AAAGAAGTGTTCGGCCATGATA -22
    
```

Insertions:

```

CTTGATGCCGTTCTTCTGGTCATCCTCATCCTGATAAACTGCAAAGAAGTGTTCGGCCATGATA wt
CTTGATGCCGTTCTTCTGGTCATCCTCtTtttGccg-ACTGCAAAGAAGTGTTCGGCCATGATA +2
TGCCGTTCTTCTGGTCATCCTCATCCTGAtctgaggAACTGCAAAGAAGTGTTCGGCCATGATA +5
    
```

Gaj T, et al, *Nature Methods*, 9, 805 (2012)より
 (-はdeletionの数、+はinsertionの数を示す)

Fig.13-1 Indel pattern of the cleavage site by ZFN

(a) **noggin TALEN**

```

TCCTAGTGA AAAACCTACC ACTGGTGGACCTTATTGAGCATCCGGATCCTA
TCCTAGTGA AAAACCTA ----- GGAGGAGAGACTTGGAG (Δ274)
GTCTTCCTG ----- // ----- GGAGGAGAGACTTGGAG (Δ197)
----- CCTTATTGAGCATCCGGATCCTA (Δ57)
TCCTAG ----- TTGAGCATCCGGATCCTA (Δ26)
TCCTAGTGA AAAACCTAC ----- TGAGC TCCGGATCCTA (Δ17, +1)
TCCTAGTGA AAAACC ----- TATTGAGCATCCGGATCCTA (Δ16)
TCCTAGTGA AA ----- ACCTTATTGAGCATCCGGATCCTA (Δ15)
TCCTAGTGA AAAACCTACCAC ----- TGAGCATCCGGATCCTA (Δ13)
TCCTAGTGA AAAACCTACC ----- TTATTGAGCATCCGGATCCTA (Δ11)
TCCTAGTGA AAAA -CTA ----- GGA TgCTTATTGAGCATCCGGATCCT (Δ11, +2)
TCCTAGTGA AAAACCTACC ACT ----- TTATTGAGCATCCGGATCCTA (Δ9)
TCCTAGTGA AAAACCTACC ----- ACCTTATTGAGCATCCGGATCCTA (Δ8)
TCCTAGTGA AAAACCTACC ACTGG ----- TATTGAGCATCCGGATCCTA (Δ7)
TCCTAGTGA AAAACCTACC tC ----- ACCTTATTGAGCATCCGGATCCTA (Δ7, +1)
TCCTAGTGA AAAACCTACC A cCT ----- ACCTTATTGAGCATCCGGATCCTA (Δ5, +1)
TCCTAGTGA AAAACCTACC ACT accacct accACCTTATTGAGCATCCGGA (Δ5, +10)
    
```

(b) **noggin ZFN**

```

GGACCTTATTGAGCATCCGGATCCTATCTATGATCCCAAGGAGAAGGATCTT
ACCTACCAC ----- // ----- AT -CCTAGGGACCAG (Δ332)
CTTATTGAGCAT ----- // ----- CCACTTTGACCCCA (Δ68)
GGACCTTATTGAG ----- CAAGGAGAAGGATCTT (Δ23)
GGACCTTATTGAGCATCCGGAT ----- CAAGGAGAAGGATCTT (Δ14)
GGACCTTATTGAG A ----- CCT -TC ----- TCCCAAGGAGAAGGATCTT (Δ14, +1)
    
```

(c) **ets1 TALEN**

```

TTACTCTGAAAGGAGTGGACTTTTCAGAAGTCTGTATGAGCGGAGCAGCA
TTACTCTGAAAGGAGTGGACTTT ----- (Δ403)
----- TATGAGCGGAGCAGCA (Δ400)
TTACTCTGAAAG ----- GAGCGGAGCAGCA (Δ25)
TTACTCTGAAAGGA ----- GTATGAGCGGAGCAGCA (Δ19)
TTACTCTGAAAGGAG ----- cGTATGAGCGGAGCAGCA (Δ18, +1)
TTACTCTGAAAGGAGTGG ----- ATGAGCGGAGCAGCA (Δ17)
TTACTCTGAAAGGAGTGGGA ----- CTGTATGAGCGGAGCAGCA (Δ12)
TTACTCTGAAAGGAGT ----- GTTCTGTATGAGCGGAGCAGCA (Δ12)
TTACTCTGAAAGGAGTGT ----- GTTCTGTATGAGCGGAGCAGCA (Δ12, +1)
TTACTCTGAAAGGAGTGT ----- GTTCTGTATGAGCGGAGCAGCA (Δ11)
TTACTCTGAAAGGAGT ----- AGTCTGTATGAGCGGAGCAGCA (Δ11)
TTACTCTGAAAGGAGTGGAC ----- TCTGTATGAGCGGAGCAGCA (Δ10)
TTACTCTGAAAGGAGTGG ----- AG cTCTGTATGAGCGGAGCAGCA (Δ10, +1)
TTACTCTGAAAGGAGTGGAC ----- TTCTGTATGAGCGGAGCAGCA (Δ9)
TTACTCTGAAAGGAGT ----- ACT ----- cAGTCTGTATGAGCGGAGCAGCA (Δ8, +1)
TTACTCTGAAAGGAGTGGACT ----- GTTCTGTATGAGCGGAGCAGCA (Δ7)
TTACTCTGAAAGGAGTGGACTT ----- TTCTGTATGAGCGGAGCAGCA (Δ7)
TTACTCTGAAAGGAGTGGACTT ----- GTTCTGTATGAGCGGAGCAGCA (Δ6)
TTACTCTGAAAGGAGTGGAC ----- aAGTCTGTATGAGCGGAGCAGCA (Δ6, +1)
TTACTCTGAAAGGAGTGGACTT ----- A cGTT aGTATGAGCGGAGCAGCA (Δ6, +2)
TTACTCTGAAAGGAGTGGACTTT ----- tGTTCTGTATGAGCGGAGCAGCA (Δ5, +1)
TTACTCTGAAAGGAGTGGACT gT ----- AAGTCTGTATGAGCGGAGCAGCA (Δ4, +1)
    
```

Lei Y, et al, *PNAS*, 109, 17484 (2012)より改変
 (Δはdeletionの数、+はinsertionの数を示す)

Fig.13-2 Indel pattern of the cleavage site by ZFN and TALEN

(a) **EMX1 site 1 full-length gRNA**

```

GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAgGGCTCCCATCACATCAACCGGTGG wild-type x35
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAG -----> Δ202
<-----
GAA -----> Δ94
<-----
GAAGCTGGAGG -----> Δ72
GAAGCTGGA ----- GG Δ56
GAAGCTGGAGGAGGAAGGGCCTGA ----- GTGG Δ39
GAAGCTGGAGGAG ----- GAAGGGCTCCCATCACATCAACCGGTGG Δ26 x2
GAAGCTGGAGGAGGAAGGGCCTGAGT ----- CCATCACATCAACCGGTGG Δ22
GAAGCTGGAGGAGGAAGGGCCTGAG ----- TCCCATCACATCAACCGGTGG Δ21 x3
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAG ----- CATCACATCAACCGGTGG Δ18
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGA ----- GCTCCCATCACATCAACCGGTGG Δ14
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGC ----- AGAAGGGCTCCCATCACATCAACCGGTGG Δ6 x3
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGC ----- AGAAGAAGGGCTCCCATCACATCAACCGGTGG Δ3 x3
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGA ----- AAGAAGGGCTCCCATCACATCAACCGGTGG Δ2 x2
GAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAACA ----- AGAAGGGCTCCCATCACATCAACCGGT +2

```

(b) **VEGFA site 3 full-length gRNA**

```

GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTGCGTGTGGGGTTGAGGGTGTGGAGCGGGGA wild-type x35
GAGGACGTGTGTGTCTGTGTG -----> Δ117
GAGGACGTGTGTGTTGG -----> Δ84
GAGGACGTGTGTGTCTGTGTG -----> Δ75
GAGGACGTGTGTGTCTGTGTG -----> Δ49
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTG -----> Δ43
GAGGACGTGTGTGTCTGTGTGGGTGAGTG -----> Δ40
GAGGACGTGTGTGTCTGTGTGAGT ----- GGGG Δ39
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGNNG -----> Δ37
GAGGACGTGTGTGTCTGTGTGGGTGAGT ----- GAGNNGNGN Δ30 x2
GAGGACGTGTGTGTCTGTGTGGGTGAGTGAG ----- TGGGGCGGGGA Δ25
GAGGACGTGTGTGTCTGTGTGGGTGAGTGAGTG ----- TGGAGCGGGGA Δ23
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTG ----- GTTGGAGCGGGGA Δ22
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTG ----- GTTGGAGCGGGGA Δ20
GAGGACGTGTGTGTCTGTGTGGGTGAG ----- TGAGGGTGTGGAGCGGGGA Δ20 x2
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTG ----- GGGCGTTGGAGCGGGGA Δ18
GAGGACGTGTGTGTCTGTGTGGGTGA ----- NNGTGGGGTTGAGGGTGTGGAGCGGGGA Δ12
GAGGACGTGTGTGTCTGTGTGGGTGAGTGA ----- GTGTGGGGTTGAGGGCGTTGGAGCGGGGA Δ8 x3
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTG ----- GGGTTGAGGGCGTTGGAGCGGGGA Δ7
GAGGACGTGTGTGTCTGTGTGG-TGAGTGTGTGTG ----- GGGTTGAGGGTGTGGAGCGGGGA Δ6
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTG ----- GNGTGGGGTTGAGGGTGTGGAGCGGGGA Δ6 x5
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTG ----- GTGTGGGGTTGAGGGTGTGGAGCGGGGA Δ4
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTGCGTGT ----- TGGGGTTGAGGGTGTGGAGCGGGGA Δ3

GAGGANGNGTGTGTCTGTGTGGGTGAGTGTGTGTGTTGGGGTTG +20
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTGTGCGTGTGGGGTTGAGGGTGTGGAGCGG +3
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTGTGCAAAAGTGTGGGGTTGAGGGTGTGGAGCGG +3
GAGGACGTGTGTGTCTGTGTGGGTGAGTGTGTGTGCGTGTGCGGGGGTTGAGGGTGTGGAGCGGG +2

```

Fu Y, et al, *Nature Biotechnology*, **32**, 279 (2014)より改変
(Δはdeletionの数、+はinsertionの数, xは頻度を示す)

Fig.13-3 Indel pattern of the cleavage site by CRISPR/Cas9