

(c) **Target 4 (EMX1): (EMX1のoff-targetサイト)**

**OT4-1**

ACCTGTACATCTGCACAAGATTGCCTTTACTCCATG	CCTTTCTTCTTCTGCTCTAACTCT	GACAATC	Wild-type x20
-----	-----	ATC	Δ64
ACCTGTACATCTGCACAAGATTGCCTTTACTCC	-----	ACAATC	Δ28
ACCTGTACATCTGCACAAGATTGCCTTTACTCCAT	-----	ACTCTGACAATC	Δ20
ACCTGTACATCTGCACAAGATTGC	-----	CTTCTGCTCTAACTCTGACAATC	Δ20
ACCTGTACATCTGCACAAGATTGCCTTTACTCCATGCCTTTCT	-----	CAATC	Δ19
ACCTGTACATCTGCACAAGATTGCCTTTACTCCA	-----	TGCTCTAACTCTGACAATC	Δ14
TCCTGTACATCTGCACAAGATTGCCTTTACTCC	-----	CTTCTTCTGCTCTAACTCTGACAATC	Δ8

(d) **Target 3 (VEGFA Site 3): (VEGFA site3のoff-targetサイト)**

**OT3-2**

GAGTGAGAGAGCGAGTGAGTG	AGTGAGTGAGTG	TGTGTGTGGGGG	GGACTCGGCTTGTGTTGTGTCGG	Wild-type x14
GAGTGAGAGAGCGAGTGAGTGAGTGAGTGA	-----	GTGTGTGGGGGGGACTCGGCTTGTGTTGTGTCGG		Δ4
GAGTGAGAGAGCGAGTGAGTGAGTGAGTGA	-----	GTGTGGGGGGGACTCGGCTTGTGTTGTGTCGG		Δ6 x2

**OT3-9**

GTGTTGGGATGCGGGAGTG	GGTGAGTGAGTGCGTGCGGGTGGC	GATGCAAGCGTGCGAATGCGTG	x173
GTGTTGGGATGCGGGA	-----	>	Δ80
GTGTTGGGATGC	-----	GCGTG	Δ50
GTGTTGGGATGCGGGAGTGGGTGAGTGA	-----	GTGGCGATGCAAGCGTGCGAATGCGTG	Δ10

GTGTTGGGATGCGGGAGTGGGTGAGTGAGTGC

AAGTGC	TGCGGGTGGCGATGCAAGCGTGCGAATGCGTG	+2
--------	----------------------------------	----

**OT3-18**

TTTCAAAGACAGTAGATCTTAAATGT	CCTCACGCACACTCACCCACAC	CATAAAAGGTGGTAAC	Wild-type x27
TTTCAAAGACAGTAGATCT	-----	TAAAAGGTGGTAAC	Δ32
TTTCAAAGACAGTAGATCTTAAATGT	-----	CATAAAAGGTGGTAAC	Δ23
TTTCAAAGACAGTAGATCTTAAATGTCCT	-----	CACATAAAAGGTGGTAAC	Δ18 x4
TTTCAAAGACAGTAGATCTTAAATGTCCT	-----	CCACACATAAAAGGTGGTAAC	Δ15
TTTCAAAGACAGTAGATCTTAAATGTC	-----	CTCACCCACACATAAAAGGTGGTAAC	Δ12
TTTCAAAGACAGTAGATCTTAAATGTCCTCA	-----	CACACTCACCCACACATAAAAGGTGGTAAC	Δ2

TTTCAAAGACAGTAGATCTTAAATGTCCTCAC

AGGCTGGAGTACAGTGGCATGATATCAGCTCACTGCAATCTCGGGCTCCCGGGTTCAAG	CCATGCACACACTCACCCACACATAAAAGGTGGTAAC	+63
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**Fig.13-4** Indel pattern of the cleavage site by CRISPR/Cas9

**Arabidopsis: mutations in 12 out of 25 sequenced clones**

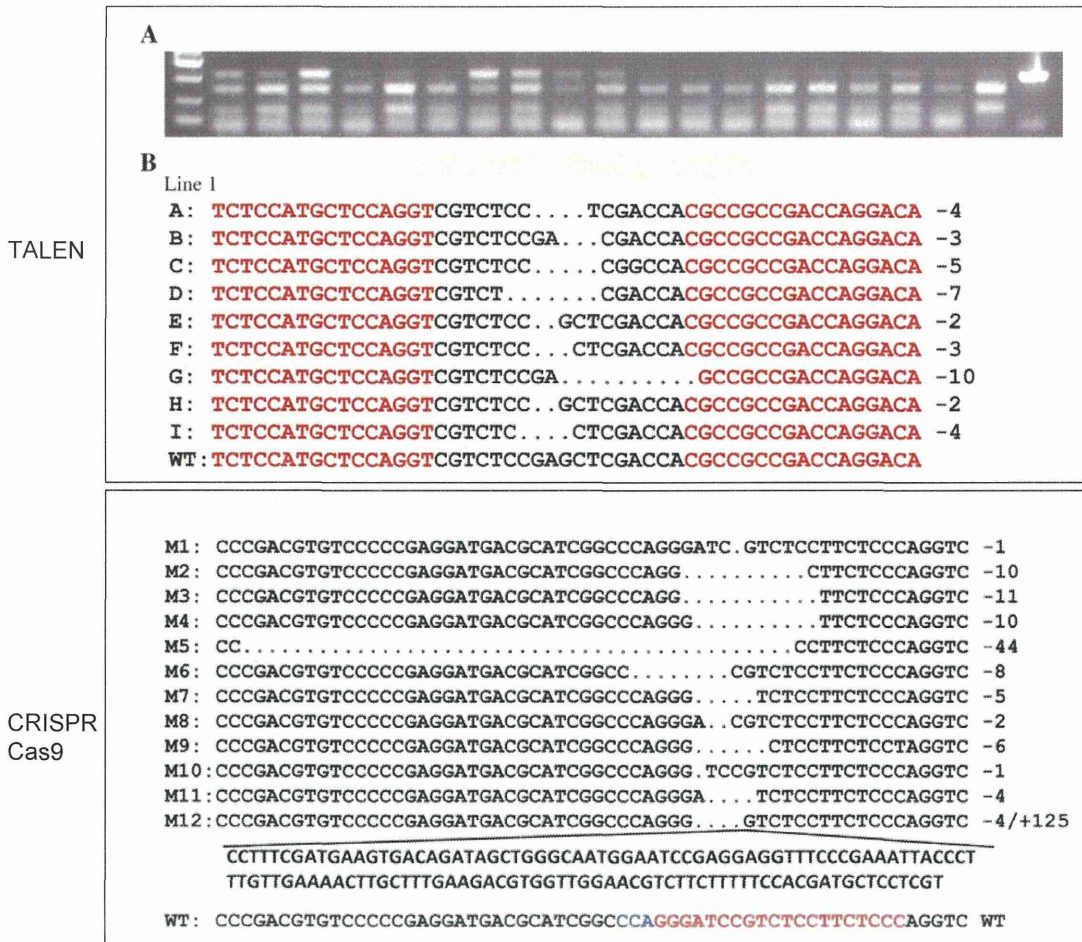
Sequence	In/Del	Freq.
CATGGAGCGCTTCAAGGTGCACATGGAGGACTAGTAAAGGAGAAGAAC		
CATGGAGCGCTTCAAGGTCCCATGGAGGACTAGTAAAGGAGAAGAAC	0 (-3, +3)	1x
CATGGAGCGCTTCAAGGTGCACATGGAGGACTAGTAAAGGAGAAGAAC	+1	1x
CATGGAGCGCTTCAAGGTGCA . . . GGAGGACTAGTAAAGGAGAAGAAC	-3	4x
CATGGAGCGCTTCAAGGTGCA . . . . . AGGACTAGTAAAGGAGAAGAAC	-5	3x
CATGGAGCGCTTCAAGGTGC . . . . . AGGACTAGTAAAGGAGAAGAAC	-6	2x
CATGGAGCGCT . . . . . GACTAGTAAAGGAGAAGAAC	-17	1x

**Tobacco: mutations in 15 out of 28 sequenced clones**

Sequence	In/Del	Freq.
CATGGAGCGCTTCAAGGTGCACATGGAGGACTAGTAAAGGAGAAGAAC		
CATGGAGCGCTTCAAGGTGCACATGGAGGACTAGTAAAGGAGAAGAAC	+1 (+1)	1x
CATGGAGCGCTTCAAGGTGCACATGGAGGACTAGTAAAGGAGAAGAAC	0 (-1, +1)	1x
CATGGAGCGCTTCAAGGTGCA . . . GGAGGACTAGTAAAGGAGAAGAAC	-3	4x
CATGGAGCGCTTCAAGGTG . . . . . TGGAGGACTAGTAAAGGAGAAGAAC	-4	1x
CATGGAGCGCTTCAAGGTGCA . . . . . GAGGACTAGTAAAGGAGAAGAAC	-4	1x
CATGGAGCGCTTCAAGGTGC . . . . . AGGACTAGTAAAGGAGAAGAAC	-5	3x
CATGGAGCGCTTCAAGGTGC . . . . . AGGACTAGTAAAGGAGAAGAAC	-6	3x
CATGGAGCGCTTCAAGGTGCA . . . . . AGGAGAAGAAC	-16	1x

Jian W, et al, *Nucleic Acid Research*, 41, e188 (2013)より改変  
 (-はdeletionの数、+はinsertionの数、xは頻度を示す)

**Fig.14-1** Indel pattern of the cleavage site by CRISPR/Cas9 in Plants (Arabidopsis and Tobacco)



Lian Z, et al, *J. Genetics Genomics*, 41, 63 (2014)より改変  
 (-はdeletionの数、+はinsertionの数を示す)

**Fig.14-2** Indel pattern of the cleavage site by TALEN and CRISPR/Cas9 in Plant (Zea mays)

### CRISPR plasmid construct

target in genome      5'-gctaggctatatttcggatGNNNNNNNNNNNNNNNNNNNGGgaattcaccgcatta-3'      (+)鎖  
 3'-cgatccgatataaagcctaCNNNNNNNNNNNNNNNNNNNCCttaagtggcgtaat-5'      (-)鎖

CRISPR基本形	ターゲット配列	PAM	ゲノム上にあるPAMは、Cas9の認識に必要であるが、sgRNAに含めない
	5'-GNNNNNNNNNNNNNNNNNN	NGG-3'	5'-GNNNNNNNNNNNNNNNNNN-----chimeric RNA (sgRNAscaffold)
	3'-CNNNNNNNNNNNNNNNNNN	NCC-5'	

Note:(-)鎖に設計する時は、向きに注意

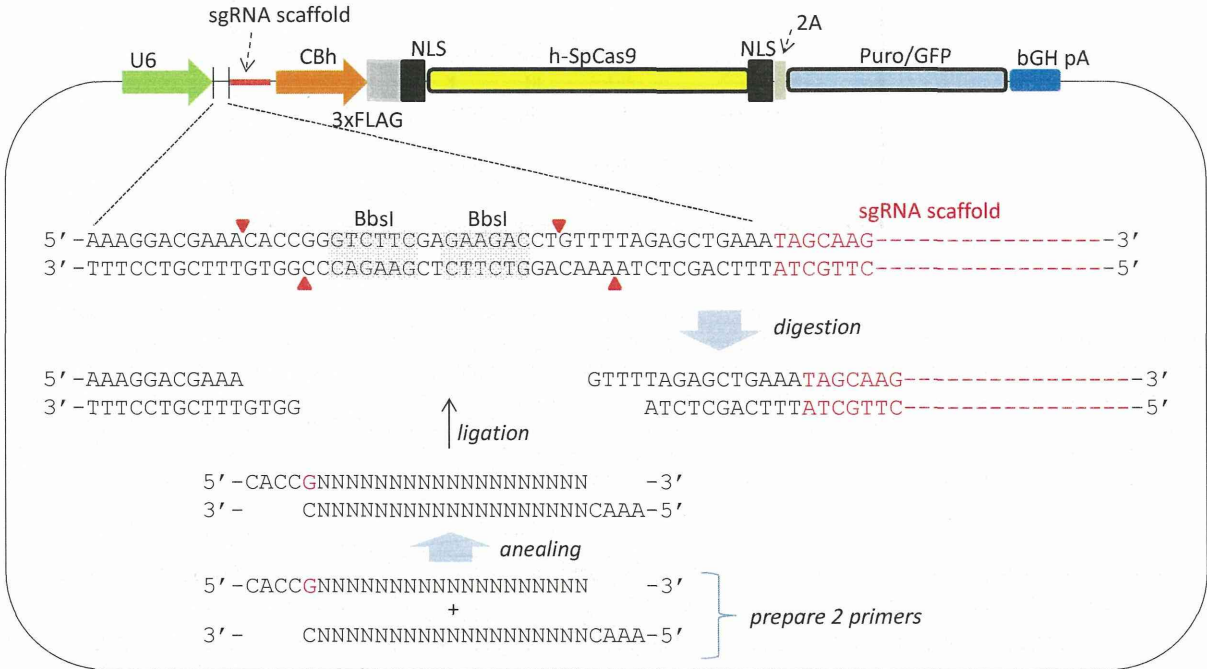


Fig.15 CRISPR/Cas9 plasmid we used in this study

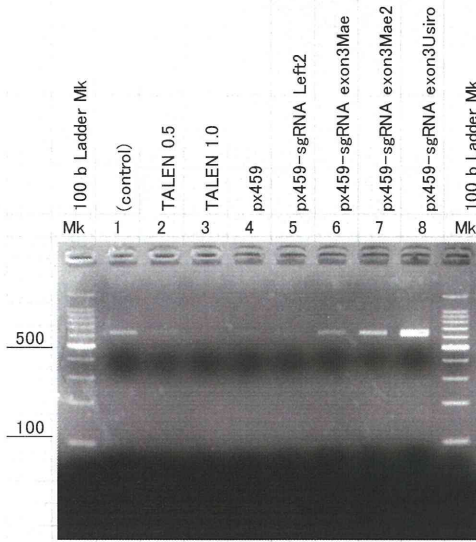


•Platinum TALENのTarget: TTTCATCCCTAGTACTG-AAAAGTCAAATTCGC-CTTAGTAGTGTTCGATA

•CRSIPR/Cas9のTarget:

Name	Length	Start	End	Strand	Nucleotide sequence	GC% in spacer	Ranking	Match-start	Match-end
U6-Rev-exon3mae2	23	20	42	minus	GAATTTGACTTTTTCAGTACTAGG	30	100	135399601	135399625
U6-Rev-exon3mae	23	116	138	plus	GTTAGCCTATGTTATGATATAGG	30	100	23972	23996
U6-Rev-left2	23	288	310	minus	GTACACAGAAGAGACATAGGGG	45	100	23800	23824
U6-Rev-exon3usiro	23	419	441	minus	GGAAACTGCTCTCTGCTATGGGG	50	100	23669	23693

SURVEYOR assay 後



TALEN spacer (talen-VR-AIF-up-A)

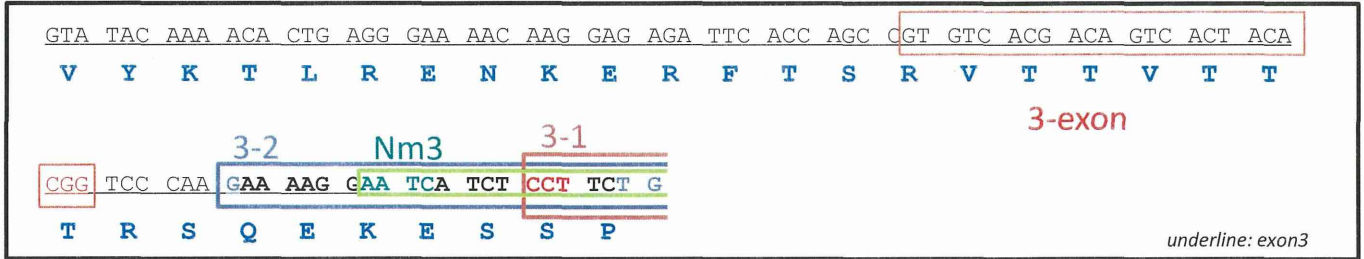
SUR-PCR4_T1-2	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-3	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-13	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-24	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-9	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-15	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-19	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-20	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-22	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-5	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-18	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-4	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-23	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-11	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-10	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-7	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-16	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-6	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-8	110	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-17	109	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
PC12HS-tetoff10	111	TTGATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	220
SUR-PCR4_T1-33	111	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	220
SUR-PCR4_T1-42	111	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	220
SUR-PCR4_T1-27	110	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-35	109	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-30	110	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-31	110	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
SUR-PCR4_T1-29	110	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	219
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SUR-PCR4_T1-37	108	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	217
SUR-PCR4_T1-45	108	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	217
SUR-PCR4_T1-44	108	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	217
SUR-PCR4_T1-26	107	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	216
SUR-PCR4_T1-48	107	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	217
SUR-PCR4_T1-34	109	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-28	109	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-46	108	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	218
SUR-PCR4_T1-43	108	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	217
SUR-PCR4_T1-47	108	CTTGTATTAGTATGCATGAAGCTCTGGGTTTCATCCCTAGTACTGAAAAGTCAAATTCGCCTTAGTAGTGTTCATAAAGGAGTAACTAGCTCAATGTAGACAAAAGCTT	217

Fig.17 AIFM1遺伝子exon3前後intron領域を標的にデザインしたsgRNAと切断活性(SURVEYOR Assay), シークエンス解析

# AIFM1 KO by disruption of exon3 (chicken DT40 exon 3 region)

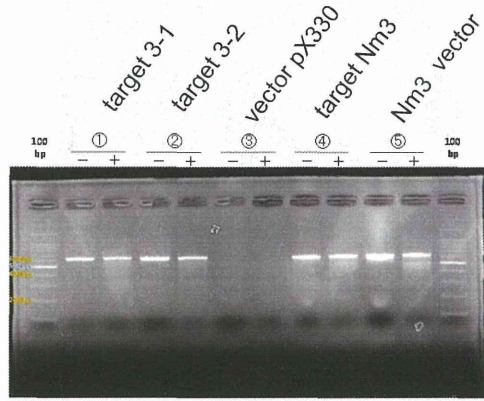
intron2

AGACAATCGGTTCTGCAGCCCAAAGACCAATGTATGCAGAAAGCTTCATTGCTTGCTCATGGAAAGCAATCGGTCTGCT  
GAGTCCCATCCTATCATAAGTAGAGCAGGTAGTTTTCTGAGTGCATTCTCCTACAGCTATGTATACATCCCCAGGTGCT  
TTTTAAACTCAGGTATATCTGGGTTTGTGGCTTTAGTTGAAAGCAATGCCTTTTCTGAGCAAGTAGGATAGACAAAAG  
TGTGTGGGGAACAGAAGCAGAATGATGCCTTGATGCTGTAACACATACAAGGTGAATGTCTTCTGTCTGTGTCCAG



GTGGGTACTTGCCCTGCAAGTTCTGGGTTTGGAGCTGTTTTCTGAGCTGCTAACTTGAGACATCCAAAACAGATAGAACG  
GAGCCTGCATGAAAACACAGTGGTAAAAAGGAGGAGGGGCCCTGCTTTGGGCTGTACCTTTTCCCAGCCTGGCAAAGAAG  
CAGTGCAGTTCCCAGCACAAAATCCTCAGTGTCTGCCATGTCCCTGTTCCATTCAAGGAGCCTCACAGAGGGAGAAATG  
AGGACAAAGTAGCAAAGCTCTTGATAATTTCTTGACACTGTATGTGGACCACAGGTCACCTCACTGAAGGTTAAGTGATT  
GTGCAGATCTAGCTTAGAGAAACCATTGCTGCAGTGTGGCACTTTGAGTCGTGCATGGAGCAGGCTTCAAGAGAAGGAG  
GGTCTTAGGAAATACAGTGGGGGAGAAGGGAAGGAGGAGAACGTATTTGGAGTGCTGTCTGCCAAAGGAGGTCCGTACA  
TGAAAAGATCTGGTGACACACTCTGT

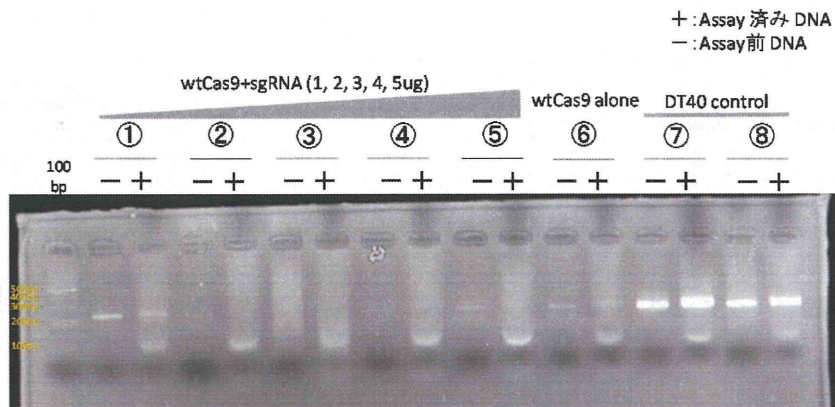
intron3



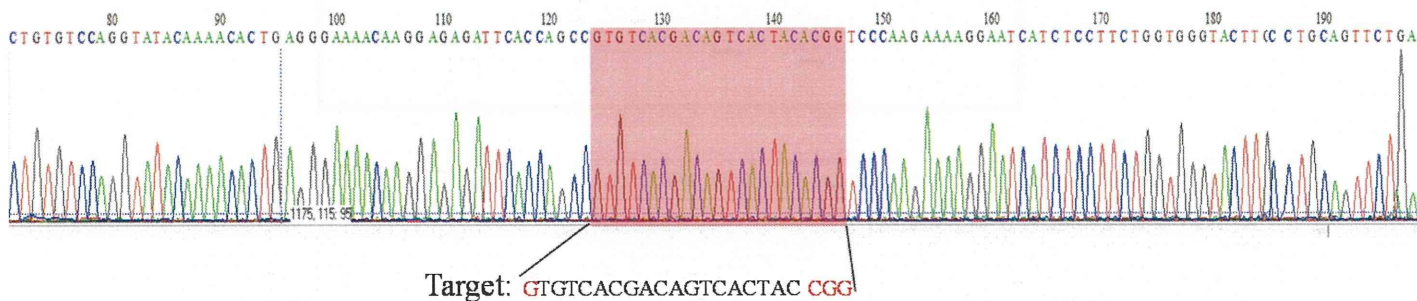
(px330: *Streptococcus pyogenes* Cas9, Nm3: *Neisseria meningitidis* Cas9)

surveyor nuclease assay

Fig.18 AIFM1遺伝子exon3-intron3にデザインしたsgRNA シークエンスと切断活性 (SURVEYOR Assay)



### sequencing 結果



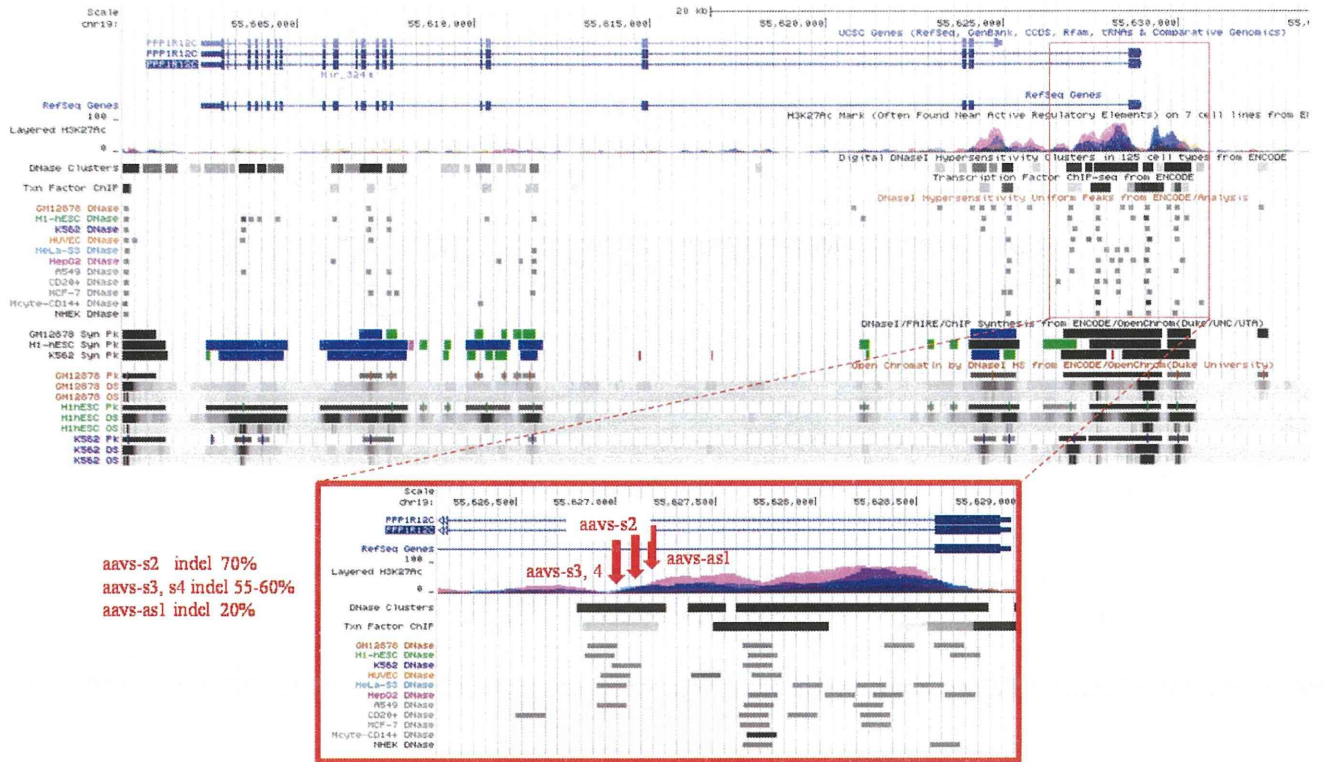
**Fig.19** *AIFM1* 遺伝子 exon3 内部にデザインした sgRNA シークエンスと切断活性 (SURVEYOR Assay) とターゲット領域のシーケンス結果

Church's group, *Nature Biotech*, 31, 833 (2013)

# PPP1R12C gene

cell type: HEK293 (Human Embryonic Kidney 293)  
target: AAVS1 locus PPP1R12C (protein phosphatase 1, regulatory subunit 12C)

(UCSC genome browser, GRCh37/hg19)



Joung's group, *Nature Biotech*, 31, 822 (2013) and *Nature Biotech*, 32, 279 (2014)

# VEGFA gene

cell type: HEK293 (Human Embryonic Kidney 293)  
target: VEGFA (vascular endothelial growth factor A)

(UCSC genome browser, GRCh37/hg19)

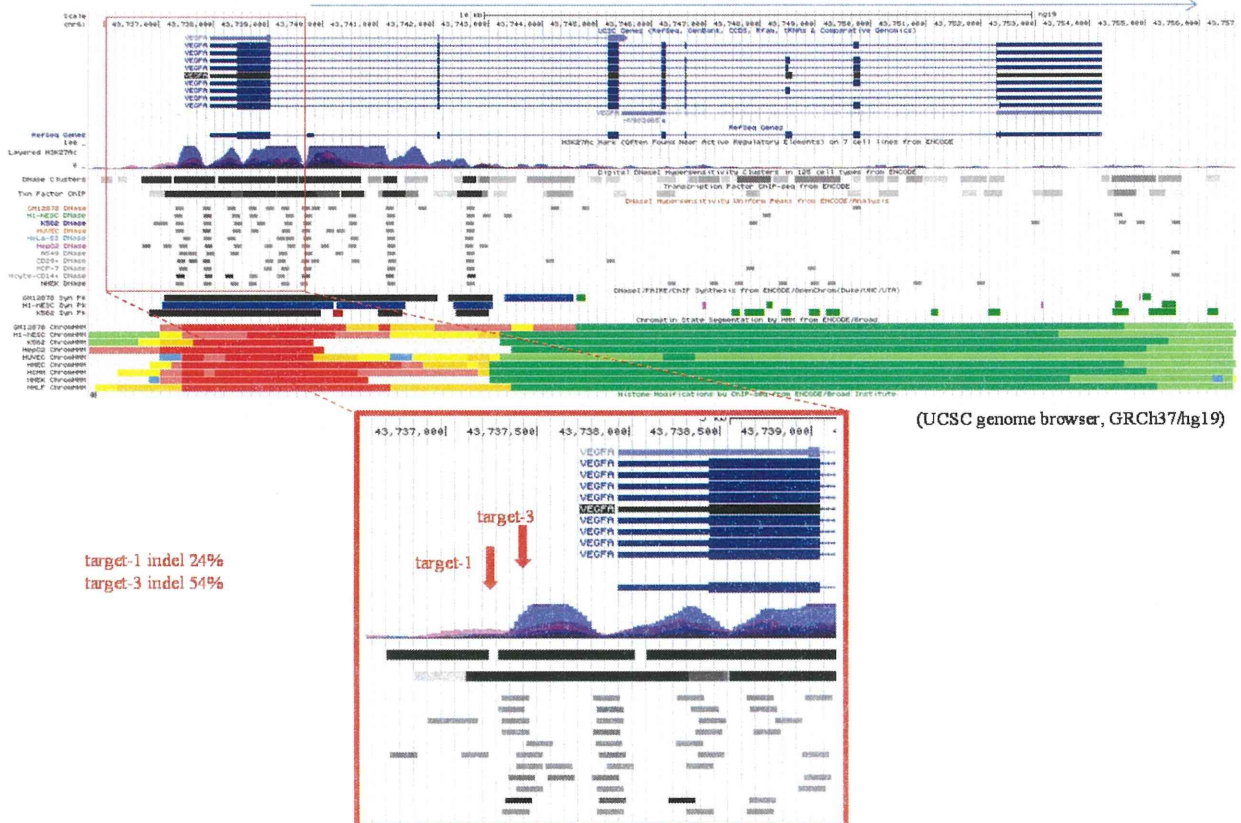


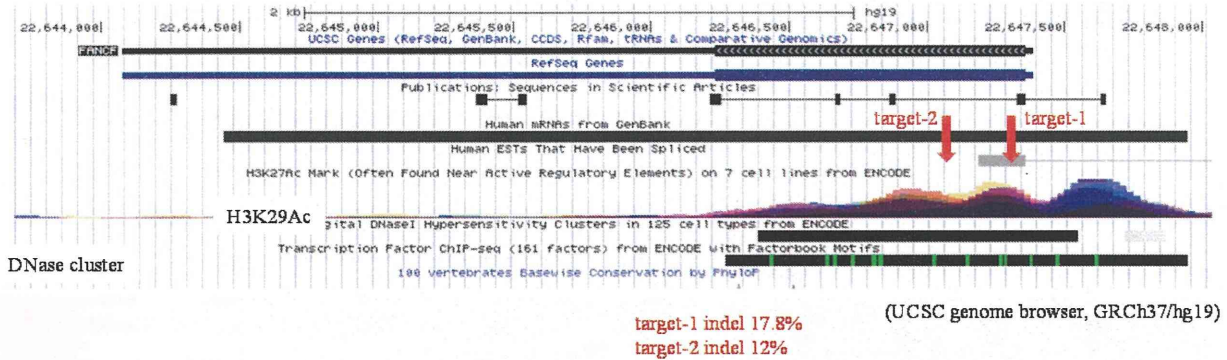
Fig.20 ENCODEデータからみた各遺伝子標的部位のゲノム構造 (continued)



Joung's group, *Nature Biotech*, advanced online doi:10.1038/nbt.2908 (2014)

cell type: HEK293& (Human Embryonic Kidney 293)  
target: FANCF (Fanconi anemia, complementation group F)

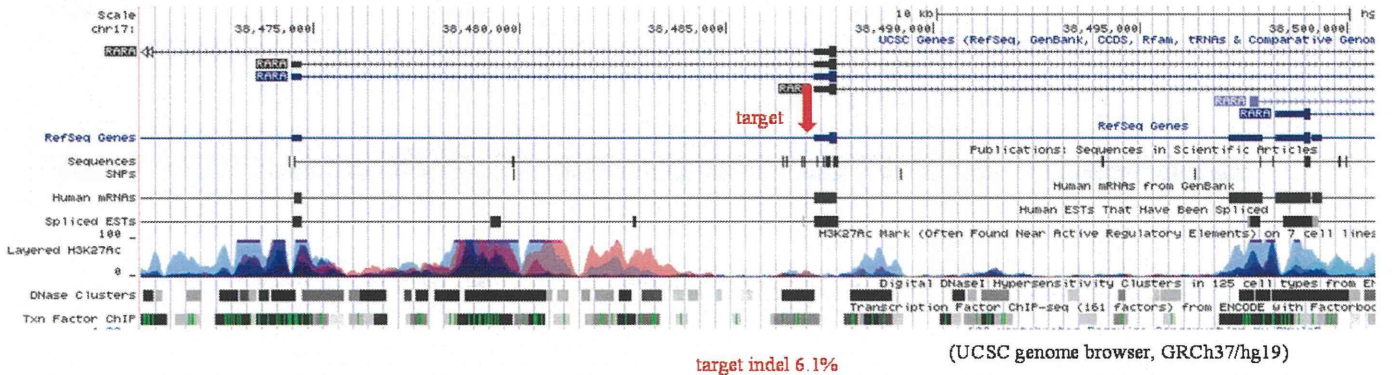
## FANCF gene



Joung's group, *Nature Biotech*, advanced online doi:10.1038/nbt.2908 (2014)

cell type: HEK293& (Human Embryonic Kidney 293)  
target: RARA (retinoic acid receptor, alpha)

## RARA gene



Joung's group, *Nature Biotech*, advanced online doi:10.1038/nbt.2908 (2014)

cell type: HEK293& (Human Embryonic Kidney 293)  
target: EMX1 (empty spiracles homeobox 1)

## EMX1 gene

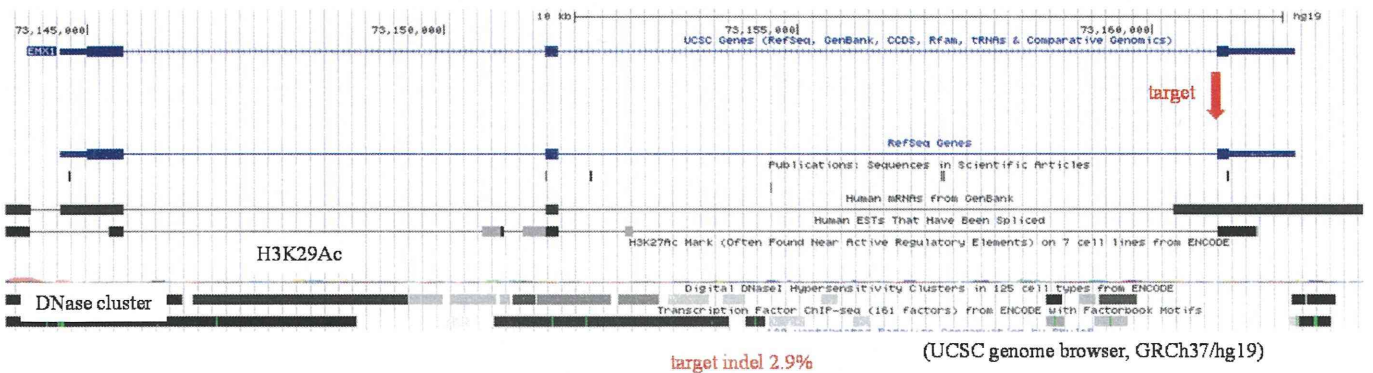


Fig.21 ENCODEデータからみた各遺伝子標的部位のゲノム構造 (continued)

# AIFM1 gene



Fig.22 ENCODEデータからみたAIFM1遺伝子exon3周辺のゲノム構造)