SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1.

CRISPR/Cas9-mediated gene disruption. (**A**) Schematic of the gene-disruption experiments. (**B**) Target sequences of CRISPR/Cas9 to *Ddx1*. The filled box indicates the untranslated region, and the open box represents the open reading frame. The CRISPR/Cas9 target sequences are colored in red, and their cleavage sites are indicated by arrowheads. The restriction enzymes used for indel confirmation are shown. (**C**) Interspecies conservation of DDX1 around the CRISPR/Cas9 target sites. The CR1 and CR2 target sites are indicated. The Q-motifs are shown in orange. (**D**) In vitro digestion of the substrate sequence by CR1. The left figure shows the expected fragment size when CR1 digests substrate DNA derived from the mouse genomic sequence. (**E**) The genomic sequence containing the CR1 target site was amplified via PCR using primers described in Supplementary Table S1. The left panel shows the expected fragment size when the amplicons derived from each clone were digested with AluI. A mutation induced by CR1 disrupts the AluI recognition site marked with an asterisk. The clone numbers and genotyping results are shown in the right panel. The largest fragment of the AluI-digested PCR product derived from wild-type and mutant alleles is indicated. C, wild-type control; Wt, wild type; He, heterozygous indel; Ho, homozygous indel. (**F**) The sequence around the CR1 target site in clone #3. The CR1 target sequence is shown in red, and the cleavage site is indicated by an arrowhead. The AluI recognition sequence is underlined. The shaded sequence represents exon 2 of *Ddx1*. A missense mutation found in one allele of clone 3 is boxed. (**G**) The genomic sequence containing the CR2 target site was amplified via PCR using primers described in Supplementary Table S1. The left panel shows the expected fragment size when the amplicons derived from each clone were digested with EcoRV. A mutation induced by CR2 disrupts the EcoRV recognition site. The clone numbers and genotyping results are shown in the right panel. The largest fragment of the EcoRV-digested PCR product derived from wild-type and mutant alleles is indicated. C, wild-type control; Wt, wild type; He, heterozygous indel; Ho, homozygous indel. (**H**) A target sequence of CRISPR/Cas9 in *Slc20a2*. The filled box indicates the untranslated region, and the open box represents the open reading frame. The target sequence of CRISPR/Cas9 is colored in red, and its cleavage site is indicated by an arrowhead. The restriction enzyme used for indel confirmation is shown. (**I**) The genomic sequence containing the CRISPR/Cas9 target site of *Slc20a2* was amplified via PCR using primers described in Supplementary Table S1. The left panel shows the expected fragment size when the amplicons derived from each clone were digested with SacII. A mutation induced by *Slc20a2*CR disrupts the SacII recognition site. The clone numbers and genotyping results are shown in the right panel. The largest fragment of the SacII-digested PCR product derived from wild-type and mutant alleles is indicated. C, wild-type control; Wt, wild type; He, heterozygous indel; Ho, homozygous indel.

Supplementary Figure S2.

Details of *Ddx1* targeting. The genomic sequences of wild-type, targeted, and FLPed *Ddx1* around exon 2 are shown. Uppercase and lowercase letters represent exons and introns, respectively. The target sequence of CRISPR/Cas9 is underlined. The arrowhead indicates the cleavage site. The exon 2 sequence upstream and downstream of the cleavage site is colored blue and red, respectively. The inserted sequence (all-in-one unit) of the targeting vector is boxed in red. The EGFP sequence without a stop codon is shown in green with underlining. The inserted residues essential for shifting the reading frame after FLP/FRT recombination are indicated by double underlining. Silent mutations were introduced at the dotted residues of the all-in-one unit to avoid CRISPR/Cas9-mediated cleavage of the inserted sequence.

Supplementary Figure S3.

Southern blot analysis of TetFE *Ddx1*cKO clones. PCR-verified non-targeted allele-negative clones were analyzed by Southern blotting as in Figure 2D. N, TetFE ESCs. Asterisks indicate F/mut clones with a large indel in the mutant allele. Clone #1 was used as TetFE *Ddx1*$^{F/F}$ ESCs in this study.

Supplementary Figure S4.
Ddx1 cKO ESCs. (**A**) Lv-a2FE-infected *Ddx1* cKO ESCs were sorted and incubated with or without 4OHT for 2 days. The expression of fluorescent proteins was analyzed via flow cytometry. (**B**) Sorted EGFP^{+} (F/F) and EGFP^{-} (-/-) cells were cultured for 2 days, and DDX1 expression was analyzed via western blotting. (**C**) DDX1 expression in *Ddx1*$^{-/-}$ ESCs was analyzed by western blotting. The arrow indicates DDX1. The asterisk shows a non-specific band found in both samples. C, control parental ESCs. (**D**) DDX1 was immunoprecipitated from *Ddx1* cKO cells using anti-FLAG agarose beads, and the associated proteins were detected via silver staining. C, control immunoprecipitation using parental cell lysate.

Supplementary Figure S5.
Generation of TetFE ESCs. (**A**) Modified SIM system for loading multiple genes into the *Gt(ROSA26)Sor* locus. Instead of the Cre/loxP-mediated gene integration method used in the original SIM system, the modified SIM system used an NHEJ-dependent knock-in mechanism catalyzed by the CRISPR/Cas9 vectors TetFE CR1 and TetFE CR2. Arrowheads represent primers used for genomic PCR. (**B**) Genomic PCR analysis of G418-resistant clones. The sizes of PCR products generated with these primer sets are shown. The upper band represents the targeted allele and the lower band represents the non-targeted allele. C, wild-type control. (**C**) Expression of fluorescent marker proteins in TetFE ESCs was analyzed via flow cytometry.

Supplementary Figure S6.
Generation of various all-in-one conditional gene knockout TetFE ESCs. EGFP-positive clones identified by fluorescence microscopy were picked up for characterization. (**A**) Targeting strategy for *Hnrnpk*. Arrowheads represent primers used for genomic PCR. The probe position used for Southern blotting is depicted in the figure. X, XcmI recognition site. (**B**) Genomic PCR analysis of the targeted clones. The sizes of PCR products generated with these primer sets are shown. The upper band represents the non-targeted allele and the lower band represents the targeted allele. C, wild-type control. (**C**) XcmI-digested genomic DNA of clones #1 and #2 was analyzed by Southern blotting. C, wild-type control. The expected fragment sizes of the non-targeted and targeted alleles are 3.8 and 4.8 kb, respectively. Clone #2 was used as *Hnrnpk*$^{F/F}$ ESCs. (**D**) HNRNPK expression in *Hnrnpk*$^{F/F}$ ESCs was analyzed via western blotting. (**E**) Expression of *Hnrnpk* mRNA in *Hnrnpk*$^{F/F}$ cells was analyzed by quantitative RT-PCR (n = 3; NS, not significant). (**F**) The purity of the sorted *Hnrnpk*$^{F/F}$ and *Hnrnpk*$^{-/-}$ cells was analyzed via flow cytometry on day 1. (**G**) The expression of HNRNPK protein in the sorted cells was analyzed via western blotting. Cells were harvested on day 1. (**H**) Targeting strategy for *Rtraf*. Arrowheads represent primers used for genomic PCR. The probe position used for Southern blotting is depicted in the figure. N, NdeI recognition site. (**I**) Genomic PCR analysis of the targeted clones. The sizes of PCR products generated with these primer sets are shown. The upper band represents the targeted allele, and the lower band represents the non-targeted allele. C, wild-type control. (**J**) NdeI-digested genomic DNA of clones #2 and #3 was analyzed by Southern blotting. C, wild-type control. The expected fragment sizes of the non-targeted and targeted alleles are 4.3 and 5.4 kb, respectively. Note clone #2 had a large indel in one allele. Clone #3 was used as *Rtraf*$^{F/F}$ ESCs. (**K**) RTRAF expression in *Rtraf*$^{F/F}$ ESCs was analyzed via western blotting. (**L**) Expression of *Rtraf* mRNA in *Rtraf*$^{F/F}$ cells was analyzed by quantitative RT-PCR (n = 3; ** P<0.01). (**M**) The purity

of the sorted *Rtraf*$^{F/F}$ and *Rtraf*$^{-/-}$ cells was analyzed via flow cytometry on day 2. (**N**) RTRAF expression in the sorted cells was analyzed via western blotting. Cells were harvested on day 2. (**O**) Targeting strategy for *Ddx39*. Arrowheads represent primers used for genomic PCR. (**P**) Genomic PCR analysis of the targeted clones. The sizes of PCR products generated with these primer sets are shown. The upper band represents the targeted allele, and the lower band represents non-targeted allele. C, wild-type control. (**Q**) The sequences around the CR target site in the three clones were analyzed. The CR target sequence is shown in red, and the cleavage site is indicated by an arrowhead. Clone #1 was used as *Ddx39*$^{F/mut}$ ESCs. (**R**) DDX39 expression in *Ddx39*$^{F/mut}$ ESCs was analyzed via western blotting. The arrowhead indicates FLAG-DDX39. Note that the anti-DDX39 antibody recognizes both DDX39 and the highly conserved paralogous protein DDX39B. The lower band detected by this antibody contains endogenous DDX39B and/or DDX39. Note that *Ddx39*$^{F/mut}$ ESCs express less DDX39 than parental cells due to loss of *Ddx39* expression from the mutated allele. (**S**) The purity of the sorted *Ddx39*$^{F/mut}$ and *Ddx39*$^{-/mut}$ ESCs was analyzed via flow cytometry on day 2. (**T**) DDX39 expression in the sorted cells was analyzed via western blotting. Cells were harvested on day 2. The arrowhead indicates FLAG-DDX39.

Supplementary Figure S7.
All-in-one cKO in HT1080 cells. (**A**) Targeting strategy for *DDX1*. Arrowheads represent primers used for genomic PCR. The probe position used for Southern blotting is depicted in the figure. N, NdeI recognition site. (**B**) Genomic PCR analysis of the targeted clones. The sizes of PCR products generated with these primer sets are shown. The upper band represents the targeted allele and the lower band represents the non-targeted allele. C, wild-type control. (**C**) Genomic DNA of PCR-verified non-targeted allele-negative clones was digested with NdeI for Southern blotting. C, wild-type control. The expected fragment sizes of non-targeted and targeted alleles are 5.3 and 6.3 kb, respectively. (**D**) DDX1 expression in *DDX1* cKO HT1080 cells was analyzed via western blotting. (**E**) Expression of *DDX1* mRNA in *DDX1*$^{F/F}$ cells was analyzed by quantitative RT-PCR (n = 3; * P<0.05). (**F**) *DDX1*$^{F/F}$ cells were transiently transfected with the codon-optimized FLP expression vector, and *DDX1*$^{F/F}$ and *DDX1*$^{-/-}$ cells were sorted based on EGFP expression. The purity of sorted *DDX1*$^{F/F}$ and *DDX1*$^{-/-}$ cells was analyzed by flow cytometry on day 1. (**G**) Expression of DDX1 protein in sorted cells was analyzed via western blotting. Cells were harvested on day 1. (**H**) Targeting strategy for *HNRNPK*. Arrowheads represent primers used for genomic PCR. The probe position used for Southern blotting is depicted in the figure. B, BsoBI recognition site. (**I**) Genomic PCR analysis of the targeted clones. The sizes of PCR products generated with these primer sets are shown. The upper band represents the targeted allele and the lower band represents the non-targeted allele. Asterisks indicate clones with a slightly higher targeted allele signal. Sequencing analysis of these clones revealed partial duplication of the right homology arm sequence in the targeted allele. They were considered to be non-targeted clones. C, wild-type control. (**J**) Genomic DNA of PCR-verified non-targeted allele-negative clones was digested with BsoBI for Southern blotting. C, wild-type control. The expected fragment sizes of non-targeted and targeted allele are 4.2 and 5.2 kb, respectively. (**K**) HNRNPK expression in *HNRNPK* cKO HT1080 cells was analyzed via western blotting. (**L**) Expression of *HNRNPK* mRNA in *HNRNPK*$^{F/F}$ cells was analyzed by quantitative RT-PCR (n = 3; NS, not significant). (**M**) *HNRNPK*$^{F/F}$ cells were transiently transfected with the codon-optimized FLP expression vector, and *HNRNPK*$^{F/F}$ and *HNRNPK*$^{-/-}$ cells were sorted based on EGFP expression. The purity of sorted *HNRNPK*$^{F/F}$ and *HNRNPK*$^{-/-}$ cells was analyzed by flow cytometry on day 1. (**N**) Expression of HNRNPK protein in sorted cells was analyzed via western blotting. Cells were harvested on day 1.

Fig.S1 Suzuki et al.

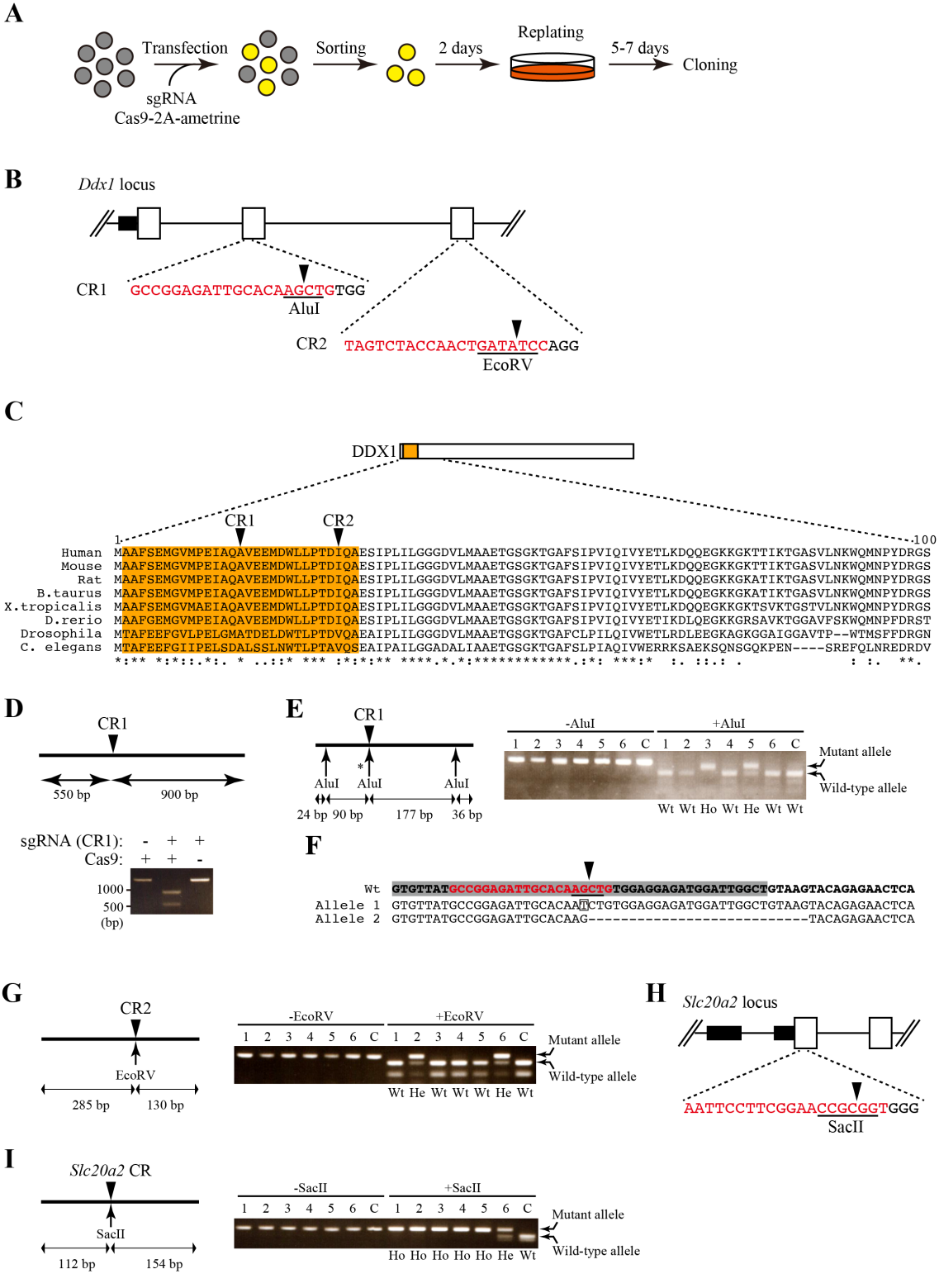


Fig.S2 Suzuki et al.

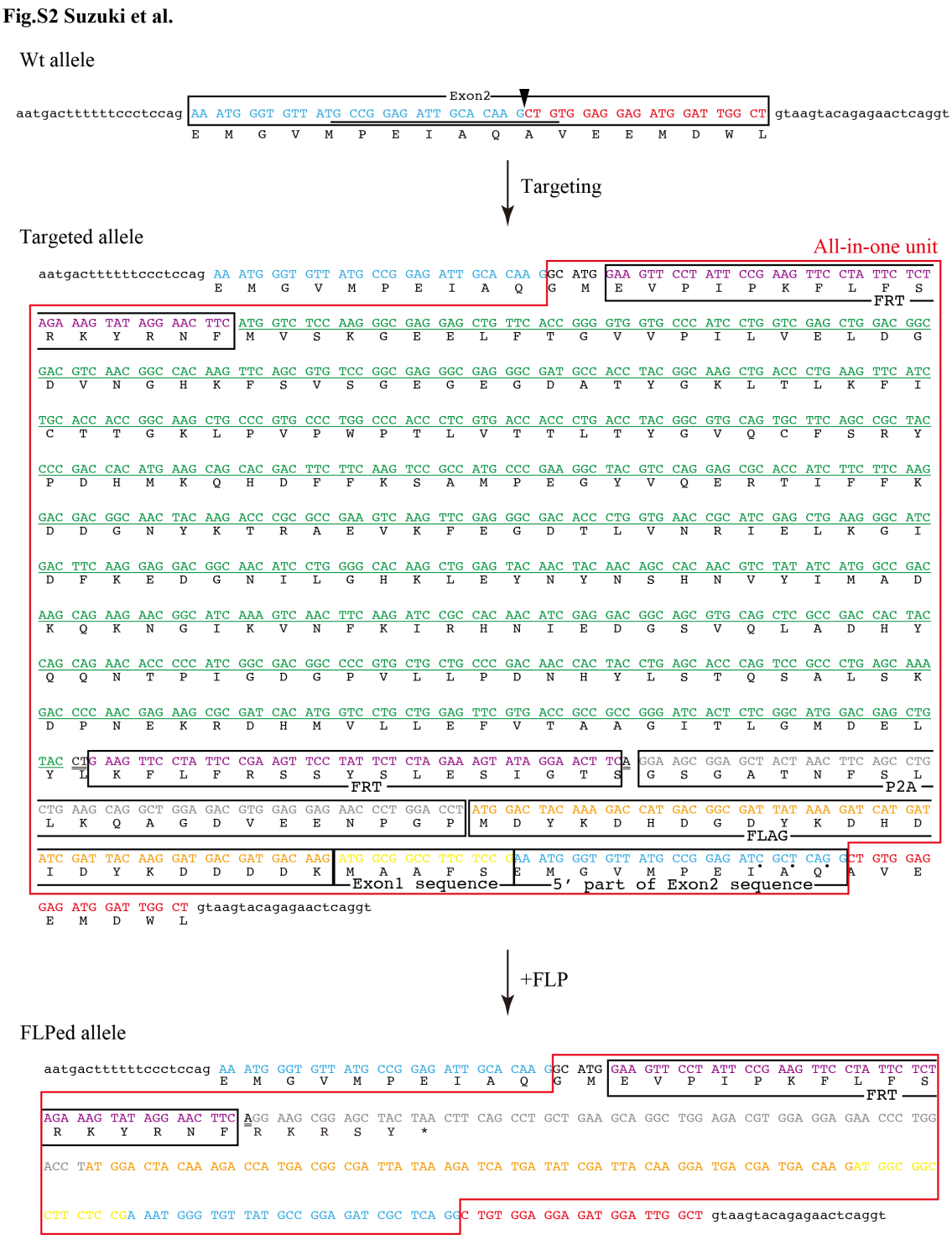
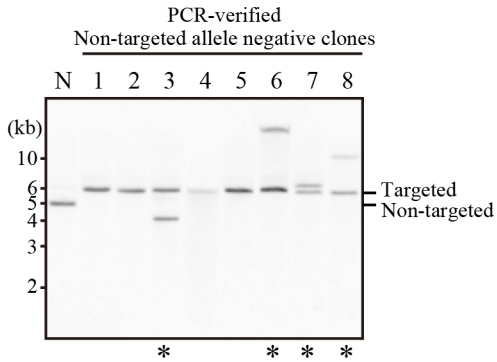


Fig.S3 Suzuki et al.



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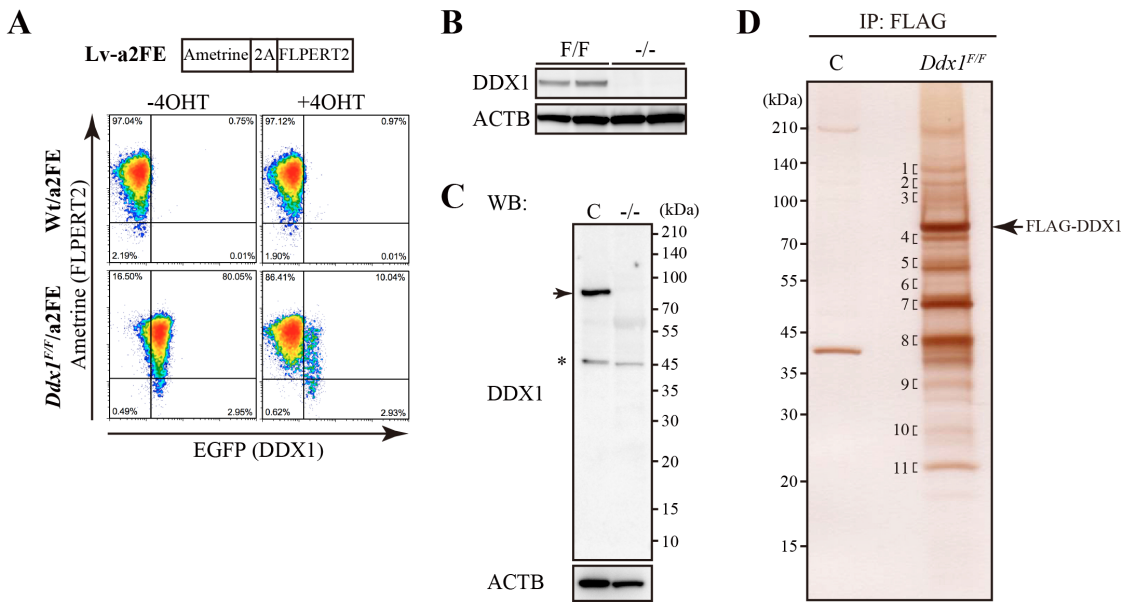

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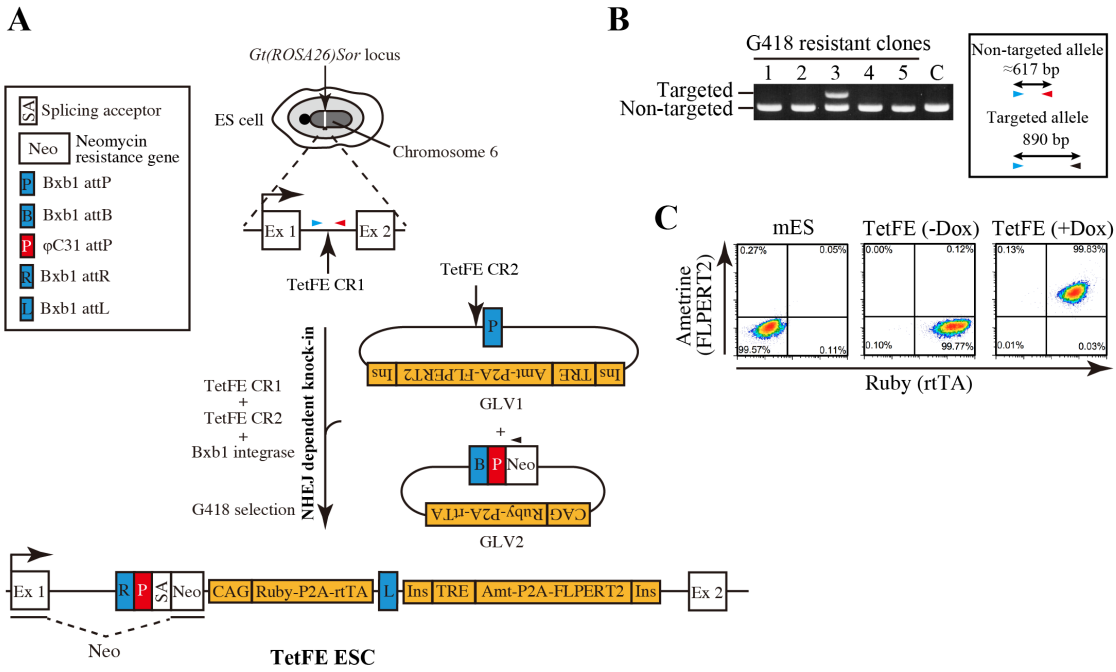

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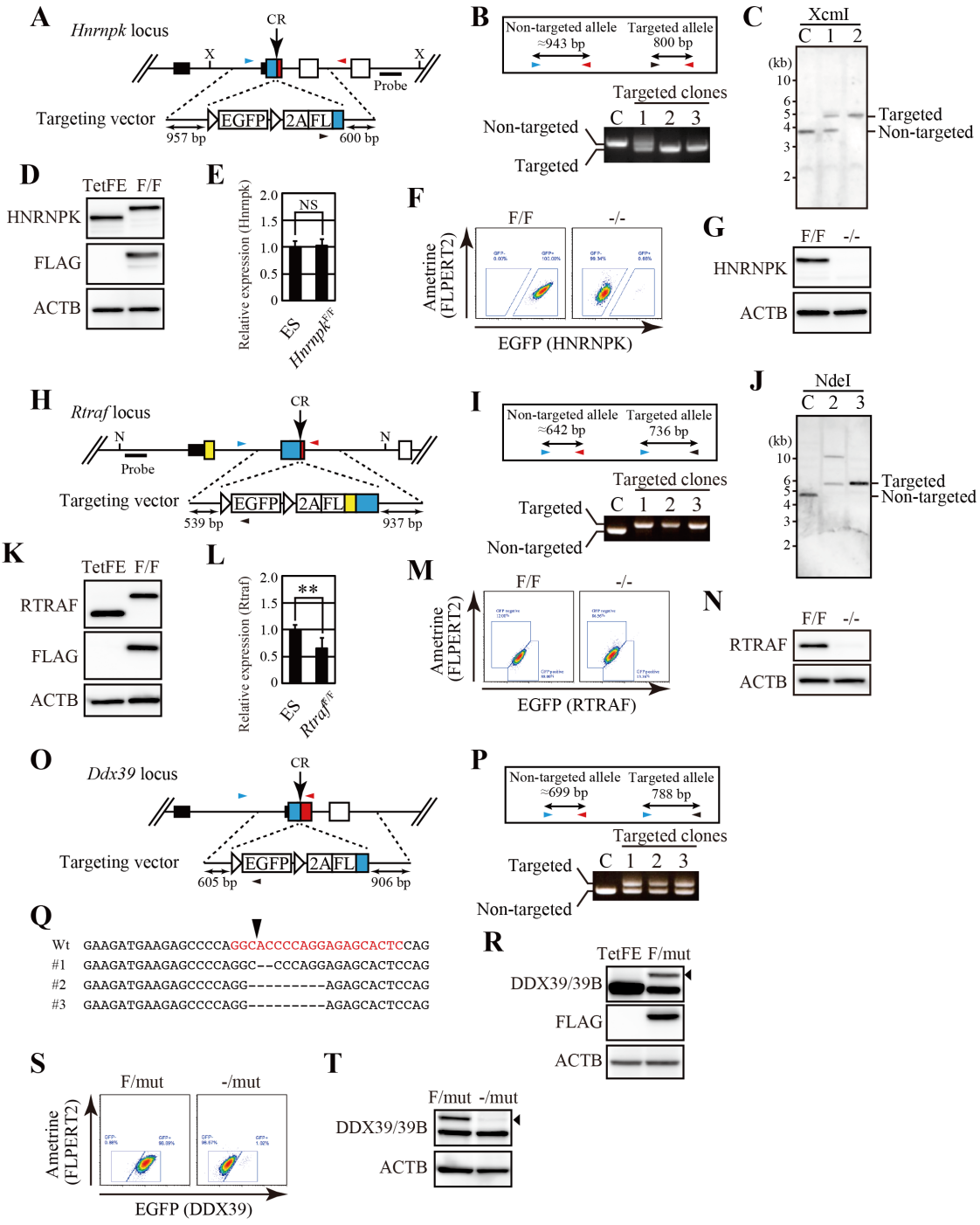

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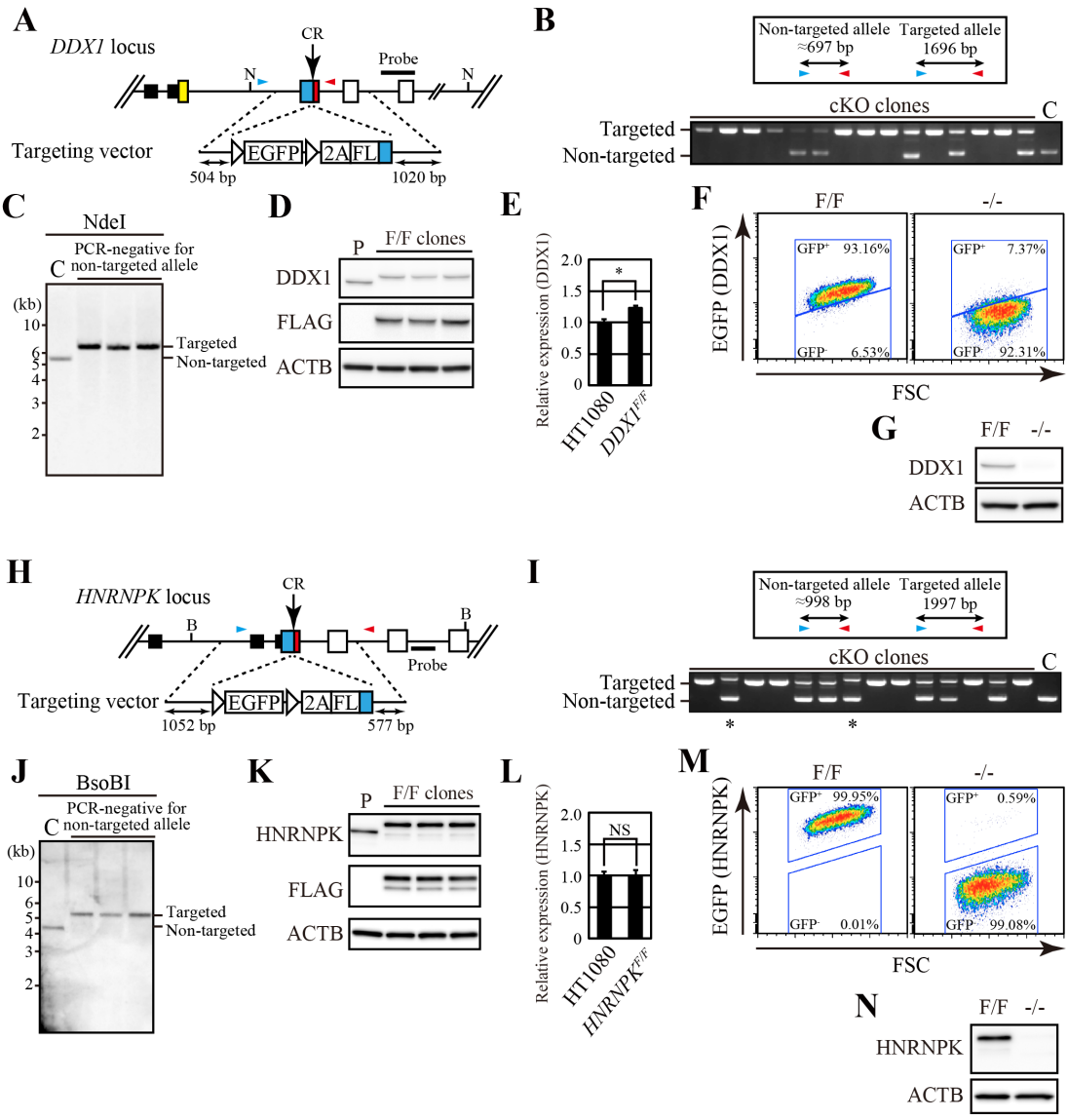

Supplementary Table S1. Primer sequences.

	Primer sequence (5′ to 3′)
Ddx1 cKO typing (5′ junction)	TAGCAACATTTTGGCTATGGCTGTC
	GTCACAATTGCACCTGAGTTCTCTGTACTTACAGC
	CTCGCCGGACACGCTGAACTTGT
Ddx1 cKO typing (3′ junction)	CAGCCTTGGAGAGAACTTAAATCTG
	ATCGATTACAAGGATGACGATGAC
	CTTGACTAGCTAACTTTACGTCAAGTG
Hrnrpk cKO typing	ACTACCGGCAGTTCTGAAATCTGC
	ATCGATTACAAGGATGACGATGAC
	ACTACTGTAGCACTAGGATGTCAC
Rtraf cKO typing	CGTCCCGGTAACTGTACACTG
	CAACGTTTACAAAAGTTCTCTGTGC
	CTCGCCGGACACGCTGAACTTGT
Ddx39 cKO typing	CCTTTCTCAACACCAAGGCAGGTAC
	TGGAGACATAAGATCCTTTGACATC
	CTCGCCGGACACGCTGAACTTGT
TetFE typing	GTTGCAATACCTTTCTGGGAGTTC
	GGAAAATGCCAATGCTCTGTCTAG
	CGCCTTGAGCCTGGCGAACA
DDX1 typing	AGTTAAGAACTCAGCAGTTCTTCAG
	CCAGGTACATTTAGGCTTTATGTAC
HNRNPK typing	GTTCAAATCAGTATTCACTGTGATCG
	ACCAGAGCCTTCAAGTCTGTTTAG
Ddx1 probe for Southern blotting	AAAGAAACCAAAGGGACATAGC
	GAGAGGTTTTTAATTTCCTTACTAGAG
Hnrnpk probe for Southern blotting	CAGTTTGCAACATGGAGCTCATG
	GGTAGACACAAAACCTGTCATGAC
Rtraf probe for Southern blotting	CCTGTGCATGTGATTAAAGCCAGATG
	TGTCCCCAAACAGTGAAGTCAG
DDX1 probe for Southern blotting	GAATATCAGCAAAACAGCGAATAG
	CTTACACCAGTTTTGCCACT
HNRNPK probe for Southern blotting	ACTACTACACGAGCAGCTTCAG
	AGCCAAGTCCTATAAATGTGCTCAC
Ddx1 target site amplification (for CR1)	TTTGCCCCAGTTCTGTGGAAGC
	ACAAATTGTATGTGGCAATCCAGCC
Ddx1 target site amplification (for CR2)	GTACAGAGAACTCAGGTGCAATTGTGAC
	AGCATTTCCACTCTTAAGTGTC
Substrate amplification for in vitro digestion by CR1	GAGATCGTCTTAGACTAGCCAAGACAG
	GATTCTTGTGTATTGGGTCACCACTCC
Slc20a2 target site amplification	CCAAAATGGCCATGGACGGGTATC
	TAGCGTTTCCACGGTCTCGTTGTAG
Cdkn1a qRT-PCR	AGATCCACAGCGATATCCAGAC
	ACCGAAGAGACAACGGCACACT
Perp qRT-PCR	GCCAGAGCCTCATGGAGTAC
	GGCCTCCAATGACTCTCAGG
Fas qRT-PCR	CCTGAATCTAGAACCTCCAGTCG
	CCTGGATTGTCATGTCTTCAGC
Pmaip qRT-PCR	TGGAGTGCACCGGACATAAC
	CAATCCTCCGGAGTTGAGCA
Plk2 qRT-PCR	ACAACAAAGTCTACGCTGCA
	GGTGCAGTAGTCTGTGAAGCT

Mdm2 qRT-PCR	GCACCTCACAGATTCCAGCT
	GCGCTCCAACGGACTTTAAC
Rpl11 qRT-PCR	ATGGCGCAAGATCAAGGGG
	GACTGTGCAGTGAACAGCAAT
Ddx1 qRT-PCR	AGTGCTCAACAAGTGGCAGA
	TGCTCTGACAACACAGACCA
Hnrnpk qRT-PCR	AAATCATCCCTACCTTGGAAGA
	TTTAAGCATTCCACAGCATCAG
Rtraf qRT-PCR	CTCCTTCAGATTCAGCGTCAT
	CATCCTGTGTTAGACGTTCCT
DDX1 qRT-PCR	GTCAAATCACAGCACTCAGGTA
	CCGGGAAGGTTCAACAATGA
HNRNPK qRT-PCR	TCGAATCTGATGCTGTGGAATG
	CCTCCTGCTAGACTCTGATGAA
Gapdh qRT-PCR	AATGTGTCCGTCGTGGATCTGA
	GATGCCTGCTTCACCACCTTCT

Supplementary Table S2. CRISPR/Cas9-mediated gene disruption.

Target gene	Cells	Analyzed clones	Indel			ND
			No indel	Hetero	Homo	
Ddx1 (CR1)	ES	31	28	2	1*	0
	NIH3T3	18	14	4	0	0
	B16F10	10	10	0	0	0
Ddx1 (CR2)	ES	23	14	9	0	0
Slc20a2	ES	24	3	1	19	1

ND, PCR product was not detected.

* The clone represents a missense mutation in one allele.

Supplementary Table S3. Analysis of targeted allele positive clones

	Clone number	Genome PCR[a]	Non-targeted allele	Indel
Ddx1 (No selection)	**1**	T/N	In-frame mutation	21 bp deletion
	2	T		
	3	T		
	4	T		
	5	T		
	6	T/N	Exon/intron destruction	108 bp deletion including intron/exon junction of exon 2
	7	T		
	8	T		
	9	T		
	10	T		
	11	T/N	In-frame mutation	21 bp deletion
	12	T/N	Frame-shift mutation	8 bp deletion
	13	T		
Rtraf (no selection)	**1**	T/N	Frame-shift mutation	1 bp insertion
	2	T		
	3	T		
	4	T		
	5	T/N	Exon/intron destruction	247 bp deletion
	6	T/N	In-frame mutation	1 bp deletion and 1 bp insertion
Ddx39 (no selection)	**1**	T/N	In-frame mutation	9 bp del
	2	T/N	Misc	Mixture of 2 clones
	3	T/N	In-frame mutation	9 bp del
Hnrnpk (no selection)	**1**	T/N	In-frame mutation	6bp deletion
Ddx1 (GFP sorted)	**1**	T/N	In-frame mutation	3 bp deletion
	2	T/N	In-frame mutation	3 bp deletion
	3	T/N	Frame-shift mutation	46 bp deletion
	4	T/N	In-frame mutation	3 bp deletion
	5	T/N	Frame-shift mutation	1 bp deletion
	6	T		
	7	T/N	In-frame stop codon in inserted seq	5 bp deletion and 428 bp insertion
	8	T		
	9	T		
	10	T/N	Frame-shift mutation	3bp deltion and 2bp insertion
	11	T		
	12	T		
	13	T		
	14	T/N	Frame-shift mutation	1 bp deletion

	15	T/N	Frame-shift mutation	1 bp deletion
	16	T		
	17	T		
	18	T		
Hnrnpk (GFP sorted)	**1**	T		
	2	T		
	3	T		
	4	T/N	In-frame mutation	9bp deletion
	5	T		
	6	T		
	7	T/N	In-frame mutation	6bp deletion
	8	T/N	In-frame mutation	9bp deletion
Human DDX1 (GFP sorted)	**1**	T		
	2	T		
	3	T		
	4	T		
	5	T/N	Frame-shift mutation	44 bp deletion
	6	T/N	Frame-shift mutation	44 bp deletion
	7	T		
	8	T		
	9	T		
	10	T/N	Exon/intron destruction	4 bp insertion + 104 bp deletion including intron/exon junction of exon 3
	11	T		
	12	T/N	Frame-shift mutation	44 bp deletion
	13	T		
	14	T		
	15	T/N	Frame-shift mutation	25 bp deletion
	16	T/N	Frame-shift mutation	44 bp deletion
	17	T		
	18	T/N	Wild-type	No indel
	19	T		
	20	T/N	Exon/intron destruction	118 bp deletion including intron/exon junction of exon 3
	21	T		
	22	T		
	23	T		
	24	T		
Human HNRNPK (GFP sorted)	**1**	T		
	2	T		
	3	T		
	4	T/N	Exon/intron destruction	23 bp del including exon/intron junction of exon 3
	5	T/N	Exon/intron destruction	23 bp del including exon/intron junction of exon 3
	6	T		
	7	T		
	8	T/N	Exon/intron destruction	21 bp deletion + 1 bp insertion including exon/intron junction of exon 3
	9	T/N	Exon/intron	82 bp deletion + 2 bp insertion

			destruction	including exon/intron junction of exon 3
	10	T		
	11	T/N	Exon/intron destruction	23 bp del including ex/int junction of exon 3 including exon/intron junction of exon 3
	12	T		
	13	T/N	Exon/intron destruction	23 bp del including ex/int junction of exon 3 including exon/intron junction of exon 3
	14	T		
	15	T		
	16	T/N	Exon/intron destruction	23 bp del including ex/int junction of exon 3 including exon/intron junction of exon 3
	17	T/N	Exon/intron destruction	26 bp del including ex/int junction of exon 3 including exon/intron junction of exon 3
	18	T		
	19	T		
	20	T		
	21	T/N	Exon/intron destruction	82 bp deletion + 2 bp insertion including ex/int junction of exon 3
	22	T/N	Exon/intron destruction	23 bp del including ex/int junction of exon 3 including ex/int junction of exon 3

a, Targeted allele positive clones were a analyzed by 3 primer PCR. Non-targeted alleles were analyzed by sequencing. T, targeted allele signal only; T/N, both targeted and non-targeted allele signal

Supplementary Table S4. DDX1-associated proteins.

Band	Gene name	Accession number	Non-redundant peptides (confidence>95%)
1	*Dhx9*	O70133	3
2	*Dhx30*	Q99PU8	2
3	*Hnrnpu*	Q8VEK3	3
4	*Prmt5*	Q8CIG8	3
	Riok1	Q922Q2	3
5	*Pabpc1*	P29341	10
	Hspa8	P63017	4
	Hnrnpm	Q9D0E1	2
	Prmt5	Q8CIG8	4
	Krt2	Q3TTY5	2
6	*Hnrnpl*	Q8R081	2
	Krt13	P08730	3
	Prmt5	Q8CIG8	3
	G3bp2	P97379	2
	Ddx5	Q61656	2
7	*Rtcb*	Q99LF4	54
8	*Fam98b*	Q80VD1	17
	Eif4a3	Q91VC3	2
	Rbmxl1	O35479	3
	Krt14	Q61781	2
	Krt5	Q922U2	3
9	*Eif3i*	Q9QZD9	2
10	*Rpl7a*	P12970	5
	Rps3a	P97351	2
	Rpl8	P62918	3
	Rps3	P62908	2
	Rps2	P25444	2
	Gemin2	Q9CQQ4	2
11	*Rtraf*	Q9CQE8	13

Supplementary Table S5. Generation of All-in-one cKO HT1080 cells

Screening	Target locus	Clones analyzed	Targeted allele positive clones	Classification			
				cKO[a]	F/mut (in-frame indel)	F/wt	Misc.
GFP$^+$ transfected cells	*DDX1*	24	24/24 (100%)	23/24 (96%)	0/24 (0%)	1/24 (4%)	0/24 (0%)
	HNRNPK	24	22/24 (92%)	22/22 (100%)	0/22 (0%)	0/22 (0%)	0/22 (0%)

a, cKO includes F/F or F/mut with a frame-shift mutation, exon/intron disruption, an in-frame stop codon, or a large indel.