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4 **Prime editing in chicken fibroblasts and primordial germ cells**

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1 **ABSTRACT**

2 CRISPR/Cas9-based genome editing technologies are revolutionizing developmental
3 biology. One of the advanced CRISPR-based techniques is prime editing (PE), which
4 enables precise gene modification in multiple model organisms. In the current study, we
5 describe a method to apply PE to the genome of chicken fibroblasts and primordial germ
6 cells (PGCs). By combining PE with a transposon-mediated genomic integration, drug
7 selection, and the single-cell culture method, we successfully generated prime-edited
8 chick PGCs. The chicken PGC is widely used as an experimental model to study germ
9 cell formation and as a vector for gene transfer to produce transgenic chickens. Such
10 experimental models are useful in the developmental biology field and as potential
11 bioreactors to produce pharmaceutical and nutritious proteins. Thus, the method
12 presented here will provide not only a powerful tool to investigate gene function in germ
13 cell development but also a basis for generating prime-edited transgenic birds.

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16 *Keywords*:

17 gene editing, prime editing, primordial germ cell, chicken

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1 **1 | INTRODUCTION**

2 Recent advances in genome editing techniques based on the CRISPR/Cas9 system
3 enable efficient and precise genomic modification of animal models. The latest
4 representative, and perhaps most useful, technology is prime editing (PE), which enables
5 genetic information to be inserted into a targeted genomic locus (Anzalone et al., 2019;
6 Chen et al., 2021). The PE system consists of two operational components, a prime
7 editor and a prime editing guide RNA (pegRNA). The prime editor is a fused protein of
8 catalytically defective Cas9 endonuclease (dCas9 nickase) and engineered reverse
9 transcriptase. The pegRNA is both an extended guide RNA containing spacer sequences
10 to recognize and navigate dCas9 to a target site and a template for reverse transcription
11 that encodes the desired edit. In comparison with previous CRISPR-based genome
12 editing techniques, PE has several advantages, including avoidance of a double strand
13 break, less constraint of the protospacer adjacent motif (PAM) location, and greater
14 versatility than the base editing system (Komor *et al.*, 2016; Rees & Liu, 2018;
15 Scholefield & Harrison, 2021). Moreover, PE enables precise and efficient homology-
16 directed repair in both mitotic and post-mitotic cells even without an additional repair
17 donor construct (Anzalone *et al.*, 2019).

18 Chicken primordial germ cells (PGCs) have been used as a model to study germ
19 line development and cell migration (Stebler *et al.*, 2004; Nakamura *et al.*, 2007;
20 Nakamura *et al.*, 2013; Murai *et al.*, 2021). In addition, because chicken PGCs can be

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1 expanded and gene-manipulated easily *in vitro* (Hong *et al.*, 1998; Macdonald *et al.*,
2 2012; Whyte *et al.*, 2015), it has been used as a valuable vehicle of gene transfer to
3 generate transgenic (TG) birds following the establishment of TG chickens by
4 transplantation of genetically modified PGCs (van de Lavoir *et al.*, 2006; Han & Park,
5 2018). TG chickens have great potential not only as a useful model for developmental
6 biology but also as a bioreactor in the agricultural and pharmaceutical fields. Currently,
7 CRISPR-based technologies, including base editing, have been used for genome
8 modification of chicken PGCs (Dimitrov *et al.*, 2016; Idoko-Akoh *et al.*, 2018; Kim *et*
9 *al.*, 2020; Lee *et al.*, 2020; Park *et al.*, 2020). However, PE has not yet been applied to
10 chicken PGCs.

11 Here, we tested PE for a transgene (*EBFP*) and endogenous gene (*DDX4*, a gene
12 encoding a DEAD box RNA helicase) in chicken fibroblasts and PGCs. By taking
13 advantage of a long-term culture system for chick PGCs and Tol2 mobile element-
14 mediated genomic integration (Sato *et al.*, 2007; Whyte *et al.*, 2015), we devised a
15 method to efficiently obtain clones of prime-edited PGCs. This method will open paths
16 to investigating molecular mechanisms underlying PGC formation and germ cell
17 development and to generating precisely gene-modified chickens.

18

19 2 | MATERIALS AND METHODS

20 2.1 | Experimental animals

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1 Fertilized chicken eggs were obtained from Fujino-Kohkaen (Fukuoka, Japan). Embryos
2 were staged according to the Hamburger–Hamilton stages (Hamburger & Hamilton,
3 1951). All animal experiments were conducted under the ethical approval of Kyushu
4 University (No. A21-167-2).

5 **2.2 | Expression vectors and primers**

6 EBFP-C1 (#54738), PX459 (#62988), BPK1520 (#65777), pU6-pegRNA-GG-acceptor
7 (#132777), pCMV-PE2 (#132775), pCMV-PEmax (#174820), and pEF1a-hMLH1dn
8 (#174824) were obtained from Addgene. pCAGGS-T2TP has been described elsewhere
9 (Sato *et al.*, 2007). For pT2A-CAGGS-EBFP, EBFP cDNA was amplified from EBFP-
10 C1 by PCR with primers (EBFP-Fw, EBFP-Rv) and integrated into a MluI-NheI site of
11 pT2A-CAGGS (Urasaki *et al.*, 2006) using a Gibson Assembly Kit (New England
12 BioLabs). To obtain pT2A-CAGGS-PEmax-ires-ZsGreen1, "PEmax" cDNA coding a
13 fusion protein of optimized dCas9 and MMLV-reverse transcriptase was amplified from
14 pCMV-PEmax using primers (PEmax-Fw, PEmax-Rv) and inserted into a MluI-EcoRI
15 site of pT2A-CAGGS-ZsGreen1 (Atsuta & Takahashi, 2016) using a Gibson Assembly
16 Kit. The pegRNAs used in this study were designed using pegFinder
17 (http://pegfinder.sidichenlab.org; Chow *et al.*, 2021). Template DNA (GFP pegRNA-1,
18 GFP pegRNA-2, DDX4 pegRNA-1, DDX4 pegRNA-2, DDX4 pegRNA-3, DDX4
19 pegRNA-4, and DDX4 pegRNA-5) for pegRNA expression were synthesized and
20 cloned into BsaI sites of pU6-pegRNA-GG-acceptor using a Gibson Assembly Kit. To

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1 obtain sgRNA-expressing PE3 plasmids (nicking vectors of the PE3 system), oligo
2 nucleotides (PE3-EGFP-1-Fw and PE3-EGFP-1-Rv; PE3-EGFP-2-Fw and PE3-EGFP-
3 2-Rv) were annealed and inserted into the BsmBI sites of BPK1520 using a Quick
4 Ligation Kit (New England BioLabs). PuroR cDNA was amplified from PX459 with
5 primers (PuroR-Fw, PuroP-Rv) and inserted into a MluI-EcoRI site of pT2A-CAGGS
6 using a Gibson Assembly Kit, resulting in pT2A-CAGGS-PuroR. To construct the
7 pT2A-U6-pegRNA-CAGGS-PuroR vector, U6-pegRNA and CAGGS-PuroR fragments
8 were amplified with primers (U6-peg-Fw, U6-peg-Rv; CAG-PuroR-Fw, CAG-PuroR-
9 Rv) from pegRNA vectors and pT2A-CAGGS-PuroR, respectively. The fragments were
10 cloned into the ApaI-MluI and MluI-BglII sites of pT2A vectors using Ligation High
11 ver.2 (TOYOBO), resulting in the construction of pT2A-U6-pegRNA-CAGGS-PuroR
12 plasmids. Sequences of the primers, template DNA for pegRNA expression, and oligos
13 for sgRNA-expressing PE3 plasmids are shown in Supplemental Table 1.

14 **2.3 | Cell culture and derivation of PGCs**

15 Chicken fibroblast-derived DF1 cells were maintained at 38°C with DMEM/F12 (Gibco)
16 containing 10% fetal bovine serum (Biowest) and 1% penicillin–streptomycin (Gibco).
17 PGCs were obtained and maintained as previously described (Whyte *et al.*, 2015), with
18 minor modifications (Chen *et al.*, 2019). PGCs were derived by transferring 1–2 μL
19 blood isolated from HH16 embryos in 300 μL of media (FAcs media) composed of
20 DMEM (Ca^{2+} free, Gibco), 100 μM $CaCl_2$, 1× B-27 supplement (Gibco), 2 mM

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1 Glutamax (Gibco), 1× NEAA (Gibco), 55 μM β-mercaptoethanol (Gibco), 1.2 mM
2 pyruvate (Gibco), 0.2% chick serum (Biowest), 0.2% ovalbumin (Sigma-Aldrich), 0.2%
3 heparin (Sigma-Aldrich), 25 ng/mL of human activin A, and 4 ng/mL human FGF2
4 (R&D). PGCs were basically maintained in the FAcs medium at 38°C.

5 2.4 | DNA transfection, FAC-sorting and PGC electroporation

6 DF1 cells were seeded in a 48 well plate and transfected at 60–70% confluency with
7 Lipofectamine 2000 (Thermo Fisher Scientific). To establish EBFP-expressing DF1
8 cells, 1 μL Lipofectamine 2000, 750 ng pT2A-CAGGS-EBFP, and 250 ng pCAGGS-
9 T2TP were transfected. One week after transfection, cells were dissociated with 100 μL
10 0.05% trypsin–EDTA solution (Gibco), and EBFP-expressing cells were collected using
11 an SH800 cell sorter (Sony). For PE in DF1 cells, 1 μL Lipofectamine 2000, 500 ng
12 prime editor plasmid, 150 ng pegRNA plasmid, 50 ng sgRNA plasmid (a nicking
13 plasmid for PE3; where indicated), and 150 ng pEF1a-hMLH1dn (where indicated) were
14 used. The efficiency of EBFP-to-EGFP conversion was estimated using an SH800 cell
15 sorter. To obtain the PEmax expressing DF1 cells, 1 μL Lipofectamine 2000, 750 ng
16 pT2A-CAGGS-PEmax-ires-ZsGreen1, and 250 ng pCAGGS-T2TP were transfected
17 and ZsGreen-positive cells were sorted using an SH800 cell sorter 1 week after
18 transfection. For the second transfection to produce *DDX4* edited cells, pT2A-U6-
19 pegRNA-CAGGS-PuroR and pEF1a-hMLH1dn were further introduced into PEmax-
20 expressing cells. The cells were maintained in the presence of 1 μg/mL puromycin

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1 (Nacalai Tesque) for 3 days, then washed to remove puromycin and further cultured to
2 100% confluency. For electroporation to PGCs, 2×10^4 PGCs were resuspended in 10
3 μL R buffer containing 1 μg DNA plasmid (750 ng pT2A-CAGGS-EBFP, 250 ng
4 pCAGGS-T2TP; 600 ng pCMV-PEmax, 300 ng GFP pegRNA-1 plasmid, 100 ng PE3-
5 EGFP-1 plasmid; 750 ng pT2A-CAGGS-PEmax-ires-ZsGreen1, 250 ng pCAGGS-
6 T2TP; 450 ng DDX4 pegRNA-4 plasmid, 100 ng PE3-DDX plasmid, 450 ng pEF1a-
7 hMLH1dn) and electroporated using a Neon transfection system (Thermo Fisher
8 Scientific) with three pulses of 1300 V for 10 ms. To select pegRNA-transfected cells,
9 PGCs were selected using puromycin for 3 days, followed by 1 week of puromycin-free
10 culture. For a clonal culture, individual cells were sorted in a 96 well plate using an
11 SH800 cell sorter after puromycin selection and were then further cultured for 28 days.
12 Genomic DNA extraction for the prime edited DF1 or PGCs was carried out with
13 NucleoSpin Tissue XS (TAKARA) according to the manufacturer's protocol. To
14 confirm the genomic edit, a portion of the *DDX4* locus (chrZ:16,929,487-16,929,972)
15 was amplified by PCR with a set of primers (DDX4-seq-Fw, DDX4-seq-Rv) and the
16 PCR products were sequenced using the DDX4-seq-Fw primer.

17 **2.5 | Embryo injection of PGCs**

18 EGFP-expressing PGCs (3000–5000 cells/μL) were injected into hearts of HH14 host
19 embryos using a glass capillary and incubated until HH26 or HH30. Gonads of the host
20 embryos were subject to immunological staining.

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1 **2.6 | Immunohistochemistry**

2 For immunological staining on sections, the following primary antibodies were used:
3 chick anti-GFP (1/1000; abcam, ab13970), rat anti-DDX4 (1/1000; (Yoshino *et al.*,
4 2016)), and rabbit anti-Sox9 (1/500, Millipore, AB5535). Immunostaining for chicken
5 embryos was carried out as previously described (Tomizawa *et al.*, 2021). The embryos
6 were fixed in 4% PFA/PBS for 3 h at 4°C, placed in a series of sucrose/PBS solutions,
7 and embedded in OCT compound (Sakura Finetek). Ten micrometer cryo-sections were
8 permeabilized with 0.5% Triton X-100/PBS for 10 min and then incubated with
9 blocking buffer (1% blocking reagent [Roche]/TNT) for 1 h, followed by incubation of
10 primary antibodies overnight at 4°C. Sections were washed with TNT buffer and
11 incubated with secondary antibodies for 5 h at 4°C. Alexa 488 anti-chicken IgY (abcam),
12 Alexa 568 anti-rat IgG, Alexa 568 anti-rabbit IgG, and Alexa 488 anti-mouse IgG
13 (Thermo Fisher Scientific) were used as secondary antibodies. Vectashield mounting
14 media with DAPI (Vector Laboratories) was used before imaging. The sex of HH30
15 gonads were determined by Sox9 positivity. For immunostaining with cultured PGCs,
16 the cells were incubated with anti-DDX4 antibody after PFA fixation and
17 permeabilization of 0.5% Triton X-100/PBS, followed by incubation of Alexa 568 anti-
18 rat IgG.

19 **2.7 | Imaging and statistical analysis**

20 Images of stained sections were obtained using a Dragonfly confocal microscope

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1 (Andor). Statistical analyses were performed with GraphPad Prism 9 software
2 (GraphPad). The live cells in Fig. 4D were counted by using an automated cell counter
3 (TC20, Bio-Rad), Counting slides (Bio-Rad), and trypan blue (FUJIFILM).
4

5 3 | RESULTS AND DISCUSSION

6 3.1 | PE is applicable to the chicken cell line

7 To test whether PE can be used in avian cells and to efficiently visualize successful
8 genomic modification by PE, we set up the EBFP–EGFP conversion system with the
9 DF1 chicken fibroblast cell line. There are two nucleotide substitutions (T199C, A437T)
10 that result in amino acid substitutions (Y66H, Y145F) between DNA sequences
11 encoding enhanced green fluorescent protein (*EGFP*) and the enhanced blue fluorescent
12 protein (*EBFP*) (Heim & Tsien, 1996) (Fig. 1A). One of the substitutions, designated as
13 Target site-1 in the present study, is responsible for the determination of the fluorescent
14 spectrum, whereas the other, Target site-2, enhances the brightness of blue fluorescence
15 (Heim *et al.*, 1994; Heim & Tsien, 1996) (Fig. 1A). Thus, we expected that, if Target
16 site-1 C^{199} was replaced with thymidine using the PE technique, a successful
17 replacement could be monitored via EGFP fluorescence. We generated the stable EBFP
18 DF1 cell line by taking advantage of Tol2 transposon-mediated genomic integration
19 (Urasaki *et al.*, 2006; Sato *et al.*, 2007) (Fig. 1B). Prime editor- and pegRNA-1 (pegRNA
20 aiming Target site-1)-expressing vectors were transfected to the EBFP cells. As a result,

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1 EGFP-expressing cells emerged 2 days after transfection, indicating that the PE
2 technique is functional in chicken cells, as observed in other organisms (PE2; Fig. 1B).
3 Editing Target site-2 generated no EGFP-positive cells, consistent with the results of a
4 previous study (Fig. 1C) (Heim & Tsien, 1996).

5 Improved versions of PE that enhanced editing efficiency by manipulating the
6 DNA repair machinery have recently been reported (Chen *et al.*, 2021). Therefore, we
7 used the EBFP–EGFP conversion system to examine which version of the PE system
8 was most effective in chicken cells (Fig. 1C). As expected, the EBFP–EGFP conversion
9 efficiency of the PE3 system (PE2 plus a nicking vector) was higher than that of the PE2
10 system (the prime editor vector plus a pegRNA expressing vector) (Fig. 1C). We further
11 tested two engineered versions of PE: PE4 and PE5 (Chen *et al.*, 2021). PE4 is composed
12 of PE2 and a dominant negative form of DNA mismatch repair protein (MLH1dn)-
13 expressing vector, whereas PE5 is a combined system of PE3 and MLH1dn. As shown
14 in Fig. 1C, the addition of MLH1dn substantially increased the conversion efficiency of
15 PE2 and PE3 (Fig. 1C), suggesting that DNA repair machinery diminishes PE efficacy
16 in chicken cells, consistent with its effect in other animal cells (Chen *et al.*, 2021). We
17 also used a PEmax vector expressing an optimized prime editor architecture, instead of
18 the basic one, in the PE4 system (referred to as PE4m) (Fig. 1C). The PE4m system
19 yielded comparable efficiency as the PE5 system even in the absence of a nicking vector
20 (Fig. 1C). Thus, the PE4m system was used in subsequent experiments.

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1 **3.2 | PE does not the affect migratory behavior of PGCs**

2 One of the major advantages of PE is that it leads to fewer off-target effects compared
3 with conventional CRISPR/Cas9, thereby rarely causing phenotypic alteration
4 (Anzalone *et al.*, 2019). Because we aimed at applying PE to chicken PGCs, we
5 investigated whether PE affected PGC character by checking the migratory capability
6 of prime-edited PGCs (Fig. 2). EGFP-positive PGCs generated using the Tol2 system
7 have been shown to migrate correctly toward the genital ridge when injected into the
8 circulation of host embryos (Macdonald *et al.*, 2012). EGFP-expressing PGCs derived
9 from EBFP-positive cells were sorted and propagated in an optimal medium (FAcs
10 medium, see MATERIALS AND METHODS), then injected into HH14 host embryos
11 (Fig. 2A, B). We found that the grafted PGCs could reach the genital ridge of HH26
12 embryos and the developing gonads of both male and female HH30 embryos (Fig. 2B).
13 These results suggest that PE does not affect the migratory behavior at least, which is
14 one of the most representative traits of chicken PGCs.

15 **3.3 | Base substitution of *DDX4* gene by PE in the chicken cell line**

16 Having verified that transgenes in the chicken genome can be modified by PE, we next
17 attempted to edit DNA sequences for endogenous genes. The *vasa* gene, which was
18 originally isolated in *Drosophila* (Schupbach & Wieschaus, 1986), encodes a DEAD
19 box RNA helicase, and chicken *vasa* homologue *cDDX4* (also referred to as *CVH*) is a
20 prominent marker gene for PGCs (Tsunekawa *et al.*, 2000). Thus, we speculated that

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1 *DDX4* could be a candidate to validate PE for an endogenous gene in chicken PGCs. We
2 first experimented with DF1 cells because of their high availability (Fig. 3). To elevate
3 editing efficiency and to concentrate gene-edited cells, we derived DF1 cells that stably
4 expressed PEmax and ZsGreen1 bicistronically (Fig. 3A). We also used a puromycin
5 selection to harvest only pegRNA-expressing cells (Fig. 3A). Considering the sequence
6 preferences of guide RNAs in the CRISPR/Cas9 system (Xu *et al.*, 2015), we designed
7 multiple pegRNAs: pegRNA-1, -2, and -3 to abrogate the start codon of *DDX4* and
8 pegRNA-4 and -5 to install a stop codon downstream of the start codon (Fig. 3B).
9 Genomic DNAs were extracted from DF1 cells transfected with PE components, and
10 the targeted regions were sequenced (Fig. 3B). We observed peaks that originated from
11 mutated alleles of the transfected cells, except in the case of pegRNA-5-transfected cells
12 (Fig. 3B). Among pegRNA-1 to -4, pegRNA-4 appeared to be efficient, and the
13 substitution (G → T) by pegRNA-4 could not be installed by base editing techniques
14 already applied to genome modification in chickens (Komor *et al.*, 2016; Lee *et al.*,
15 2020; Porto *et al.*, 2020). Therefore, pegRNA-4 was chosen to edit DDX4 in PGC in
16 subsequent experiments.

17 **3.4 | Development of a method to isolate prime-edited PGC clones**

18 Lastly, to isolate desired mutant PGC clones, we established a combined method of
19 PE4m and a single-cell culture system (Fig. 4). PGCs transfected with both PEmax and
20 pegRNA-4 vectors were obtained by taking advantage of FAC-sorting and puromycin

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1 selection, as performed with DF1 (Fig. 4A). After we confirmed the substitution, we
2 isolated the PGCs individually and expanded them in the FAcs medium for 4 weeks
3 until sequencing analyses (Fig. 4B). Among 84 clones obtained, 7 clones had the desired
4 substitution (8.3%), whereas the substitution was not detected in 14 clones (16.7%; Fig.
5 4B). We observed nucleotide substitutions in the majority of the clones; however, most
6 of them were incomplete (65.5%) or undesired (16.7%) edits (Fig. 4B). Nevertheless,
7 our method successfully identified and isolated the PGC clones that harbored the precise,
8 desired substitution.

9 We further examined whether *DDX4* was indeed knocked out by inserting a stop
10 codon into the locus and whether the stop codon affected the proliferation of PGCs. By
11 using the antibody that specifically recognizes the N-terminus of *DDX4* (Raucci *et al.*,
12 2015, Yoshino *et al.*, 2016), we found that *DDX4* proteins were not translated in mutant
13 cells, indicating the successful disruption of *DDX4* (Fig. 4C). To assess whether *DDX4*
14 played a pivotal role in PGC proliferation, we counted the number of *DDX4*-KO cells.
15 No significant difference was observed in the cell numbers between the unedited and
16 edited groups (Fig. 4D), implying that *DDX4* is dispensable for mitosis of chicken PGC
17 even though it is essential for meiotic processes during germ cell specification in
18 multiple species (Kuramochi-Miyagawa *et al.*, 2010; Ewen-Campen *et al.*, 2013;
19 Hartung *et al.*, 2014; Taylor *et al.*, 2017).

20 We successfully applied the PE technique to the chicken fibroblast cell line and

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1 PGCs. Combined with Tol2 and the single-cell culture systems, this application enabled
2 us to obtain the desired mutant PGC clones. The proposed method would provide precise
3 genome editing in PGCs and enable tests for gene function in PGC development in both
4 *in vitro* and *in vivo* settings. Furthermore, because the PGC-mediated germline
5 transmission system is widely used to generate transgenic chickens (van de Lavoir *et al.*,
6 2006; Macdonald *et al.*, 2010; Macdonald *et al.*, 2012; Oishi *et al.*, 2016; Kim *et al.*,
7 2020; Lee *et al.*, 2020; Park *et al.*, 2020), our method could underlie potential
8 applications for the efficient genomic modification of chickens.
9

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16

17 **AUTHOR CONTRIBUTIONS**

18 **Yuji Atsuta:** study design; formal analysis; investigation; methodology; writing-
19 original draft; writing-review & editing; funding acquisition. **Katsuta Suzuki:**
20 investigation; methodology; writing-review & editing. **Haruna Yaguchi:** investigation;

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1 methodology; writing-review & editing. **Daisuke Saito:** investigation; methodology;

2 writing-review & editing; funding acquisition. All authors reviewed and approved the

3 manuscript.

4

5 CONFLICTS OF INTEREST

6 No conflicts of interest are declared.

7

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1 **Figure legends**

2 **FIGURE 1**

3 **Prime editing for transgenes in chicken fibroblast cells**

4 (A) Target sites for EBFP–EGFP conversion by prime editing (PE). PAM sequences are
5 underlined. (B) EBFP-expressing DF1 cells were transfected with prime editor- and
6 pegRNA-1 (targeting site-1)-expressing plasmids (PE2 plasmids), and a small fraction
7 of the cells started showing EGFP fluorescence (arrowheads). The fluorescence was
8 validated by FACS. (C) Comparison of different PE conditions for the EBFP/EGFP
9 conversion in DF1 cells (*n* = 14 wells for each condition). PE3 vectors are nicking
10 plasmids. The numbers show *P* values obtained using ordinary one-way ANOVA. Scale
11 bars: 200 μm in (B, C).

12

13 **FIGURE 2**

14 **Grafted prime-edited PGCs migrate correctly to the genital ridge in chicken**
15 **embryos**

16 (A) *EBFP* genes in EBFP-expressing PGCs were converted to *EGFP* by prime editing
17 (PE4m). (B, C) The PEed EGFP-expressing PGCs were sorted and subsequently
18 transplanted into HH14 embryos. The grafted cells were found at the genital ridges of
19 HH26 (B; *n* = 4 embryos) and HH30 male and female embryos (C; *n* = 3 for each sex).
20 Scale bars: 50 μm in (A), 100 μm in (B), 200 μm in (C).

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1

2 **FIGURE 3**

3 **Prime editing for an endogenous gene in chicken fibroblast cells**

4 (A) Schematics for *DDX4* locus and strategy for editing *DDX4* in DF1 cells. The *DDX*
5 gene lies at chrZ: 16,929,379–16,960,987. Cells that stably express PEmax and
6 ZsGreen1 (ZsG) were established by taking advantage of Tol2-mediated genomic
7 integration, and the ZsG^{+} cells were FAC-sorted. PegRNA-transfected cells were further
8 selected by puromycin. After the selection, DNAs extracted from transfected cells were
9 subject to Sanger sequencing. (B) Chromatograms of DNA sequences coding for the
10 first three codons of *DDX4*. PegRNAs were designed to abrogate the first codon ATG
11 (pegRNA-1, -2, and -3) or to generate a stop codon after ATG (pegRNA-4 and -5). Peaks
12 for the edited nucleotides are indicated by arrowheads. Scale bar: 100 μm in (A).

13

14 **FIGURE 4**

15 **Isolation of the prime-edited PGC clones**

16 (A) PEmax and ZsGreen-expressing PGCs were generated and collected using a cell
17 sorter. After transfection of pegRNA-4 vector and puromycin selection, DNAs were
18 extracted from the transfected PGCs and sequenced to validate the successful edit (red
19 arrowhead). (B) Schematic of experiments for identifying the edited PGC clones.
20 Individual cells were isolated using a sorter and cultured for 28 days before sequencing.

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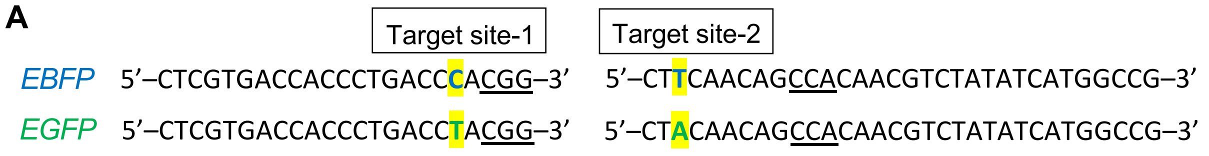
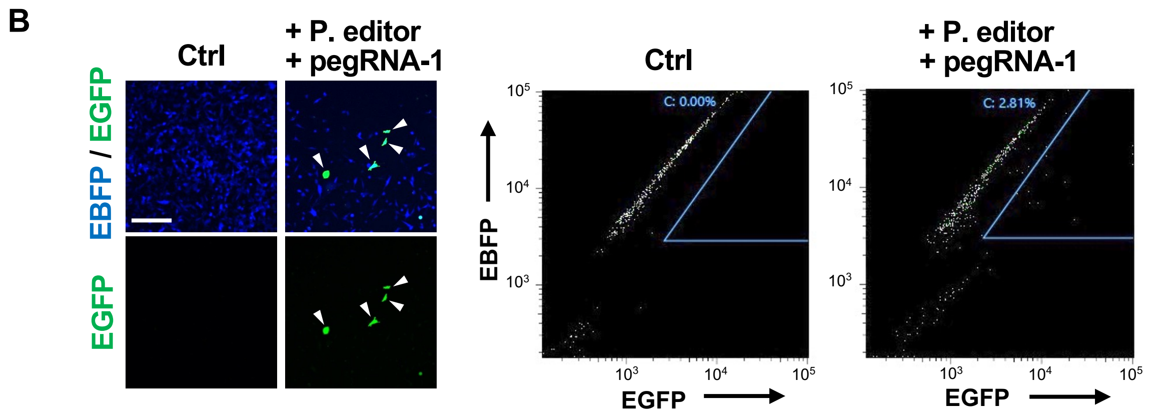
1 Traces represent completely, incompletely, and unwantedly edited DNA sequences
2 ("Completed," "Incompleted," and "Unwanted," respectively). The pie chart shows the
3 percentage of each genotypic group. (C) "Unedited" and "Completed" PGCs were
4 immunostained against DDX4. DDX4 proteins were not detected in mutated cells. (D)
5 Cell numbers were measured 28 days after the single-cell isolation (n = 7 wells for
6 "completed," n = 7 wells for "incompleted," n = 14 wells for "unedited"). The numbers
7 indicate P values obtained using an ordinary one-way ANOVA. Scale bars: 100 μm in
8 (A, B), 20 μm in (C).
9





C

Plasmids	Conditions						
P. editor	+	+	+	+	+	+	-
P. editor-max	-	-	-	-	-	-	+
pegRNA-1	-	-	+	+	+	+	+
PE3-1 (nicking)	-	-	-	+	-	+	-
pegRNA-2	-	+	-	-	-	-	-
PE3-2 (nicking)	-	+	-	-	-	-	-
hMLH1dn	-	-	-	-	+	+	+

EBFP/EGFP

EGFP

0.0008

>0.9999 0.0161 <0.0001 0.0322 >0.9999

EGFP+ (%)

20 15 10 5 0

Ctrl PE3 (peg2) PE2 PE3 (peg1) PE4 PE5 PE4m

Fig.1 Atsuta et al.

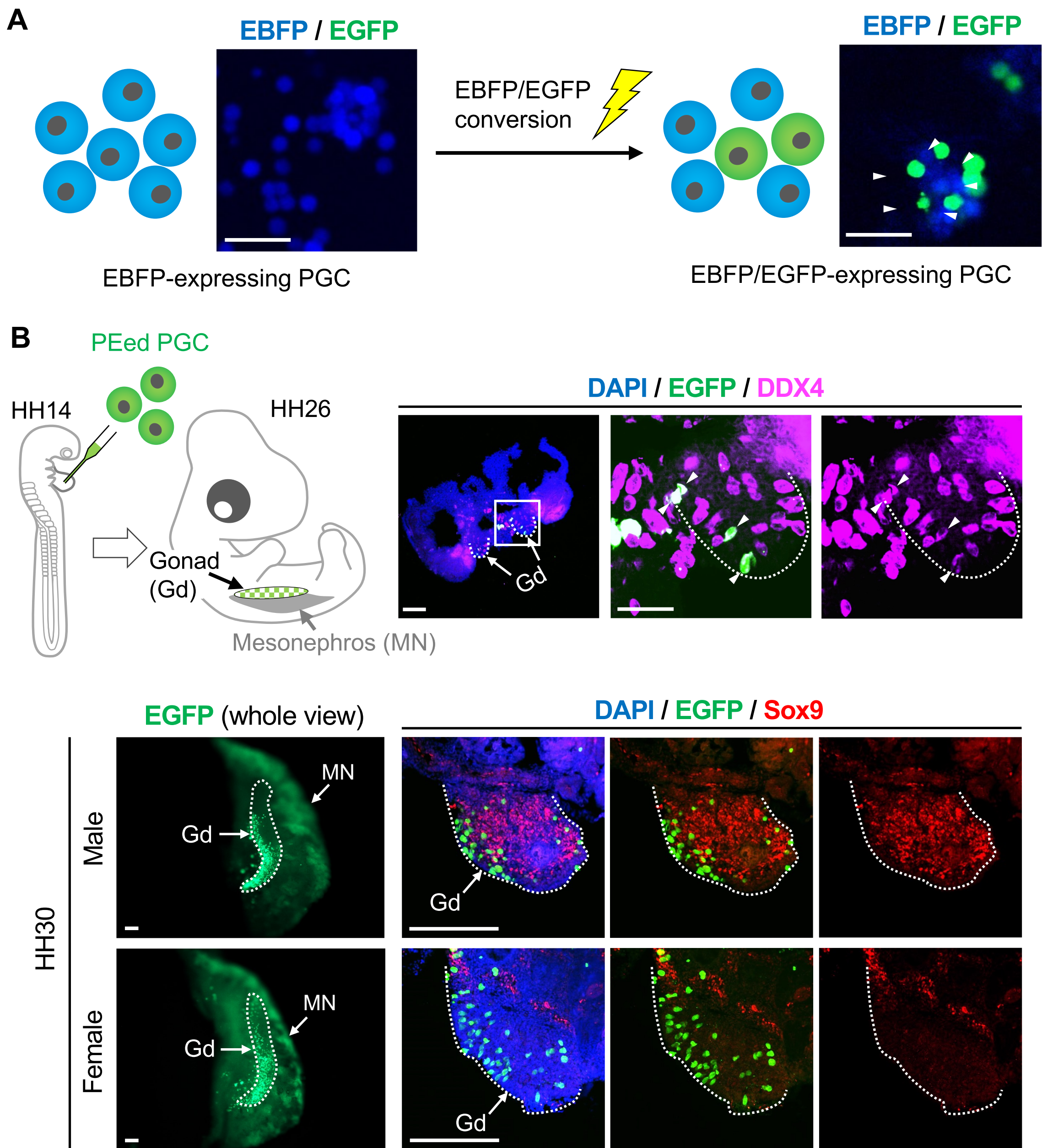
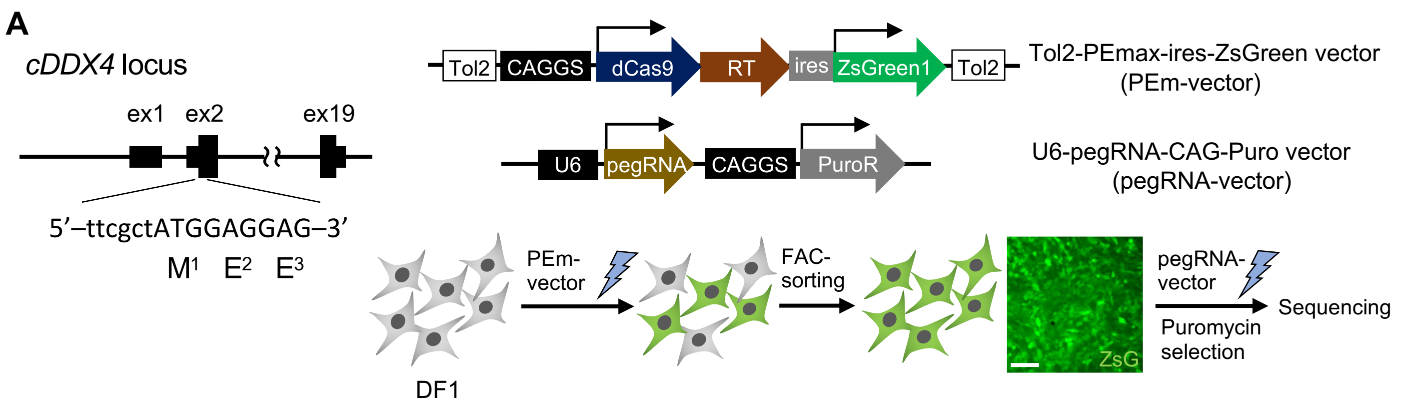


Fig.2 Atsuta et al.



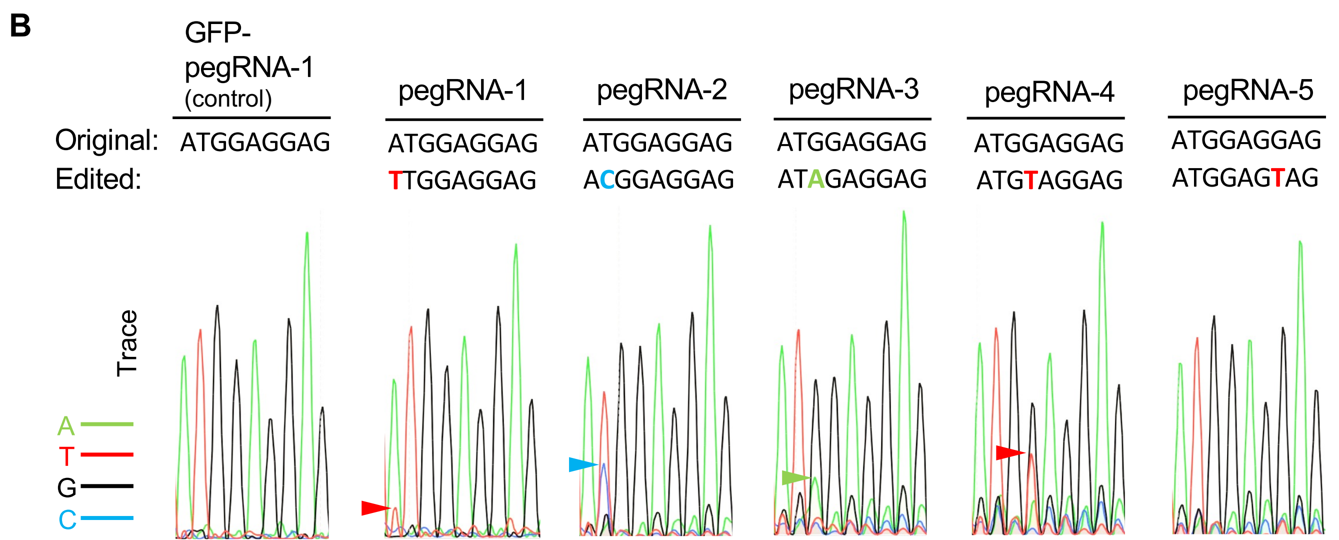
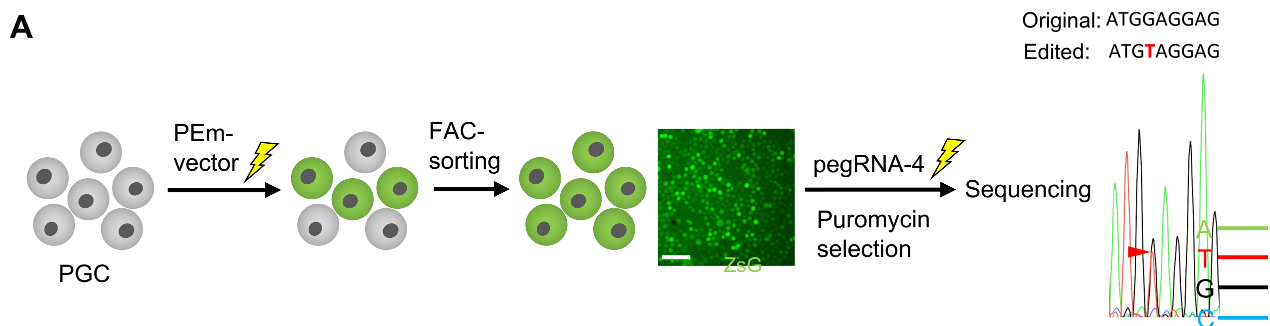
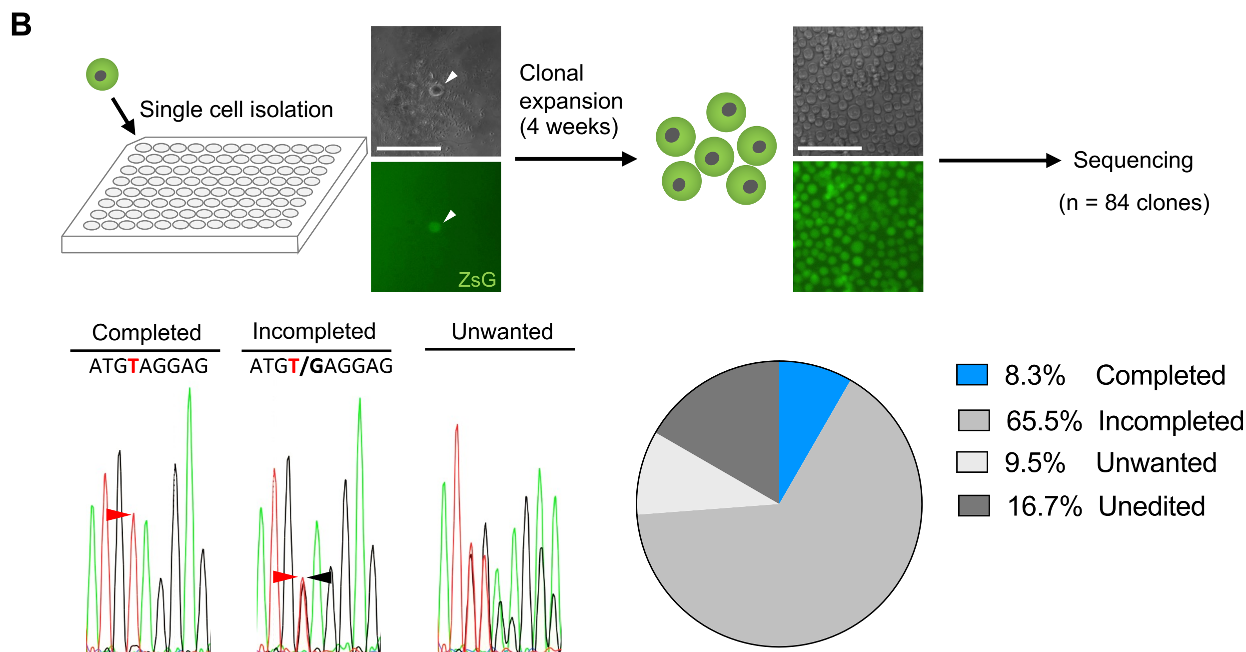
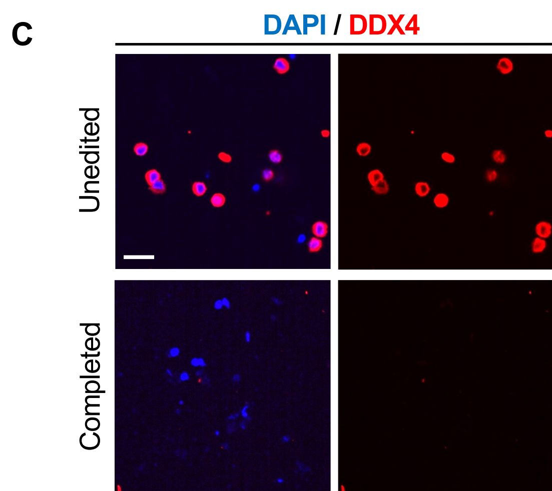


Fig.3 Atsuta et al.







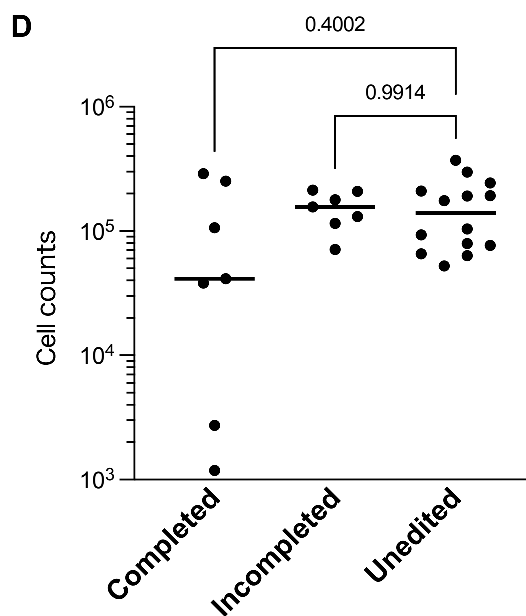


Fig.4 Atsuta et al.

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Table S1-Sequences of primers and pegRNA templates

Name	Sequence (5'-3' direction)
EBFP-Fw	TTTGGCAAAGAATTAGCCACCATGGTGAGCAAGG
EBFP-Rv	ATCCCGGGCTCGAGGTTACTTGTACAGCTCGTCCATGC
PEmax-Fw	TTTGGCAAAGAATTAGCCACCATGAAACGGACAG
PEmax-Rv	ATCCCATCTAGAATTTTAGTCCAGCTTCACTCTCTT
PuroR-Fw	TTTGGCAAAGAATTAATGACCGAGTACAAGCCCAC
PuroR-Rv	GGAAAAAGATCTGATAATTCTCAGGCACCGGGCTT
CAG-PuroR-Fw	ATAACGCGTCGGGGTCATTAGTTCATAGC
CAG-PuroR-Rv	GGCAGAGGGAAAAAGATCTG
U6-peg-Fw	ATAGGGCCCGAGGGCCTATTTCCCATGAT
U6-peg-Rv	ATAACGCGTATCAGCTTGGGCTGCAGAAA
DDX4-seq-Fw	AACCACAATGGAGCCATAGC
DDX4-seq-Rv	AAAACCGTTGCCTCTCTGTAC
PE3-EGFP-1-Fw	CACCGCTGCACGCCGTAGGTCAGGG
PE3-EGFP-1-Rv	AAACCCCTGACCTACGGCGTGCAGC
PE3-EGFP-2-Fw	CACCGGCATCGACTTCAAGGAGGA
PE3-EGFP-2-Rv	AAACTCCTCCTTGAAGTCGATGCCGC
GFP pegRNA-1	GTGGAAAGGACGAAACACCGCTCGTGACCACCCTGACCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCACTGCACGCCGTAGGTCAGGGTGGTTTTTTTAAGCTTGGGCCG
GFP pegRNA-2	GTGGAAAGGACGAAACACCGCGGCCATGATATAGACGTTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACTACAACAGCCACAACGTCTATATCATGGTTTTTTTAAGCTTGGGCCG
DDX4 pegRNA-1	GTGGAAAGGACGAAACACCGTGGGCTGCTGGCATTCGCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTCCTCCTCCAAAGCGAATGCCAGCATTTTTTTAAGCTTGGGCCG
DDX4 pegRNA-2	GTGGAAAGGACGAAACACCGCTGCTGGCATTCGCTATGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAGTCCTCCTCCGTAGCGAATGCCATTTTTTTAAGCTTGGGCCG
DDX4 pegRNA-3	GTGGAAAGGACGAAACACCGCTGCTGGCATTCGCTATGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAGTCCTCCTCTATAGCGAATGCCATTTTTTTAAGCTTGGGCCG
DDX4 pegRNA-4	GTGGAAAGGACGAAACACCGCTGCTGGCATTCGCTATGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAGTCCTCCTACATAGCGAATGCCATTTTTTTAAGCTTGGGCCG
DDX4 pegRNA-5	GTGGAAAGGACGAAACACCGCTGGCATTCGCTATGGAGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCCCCAGTCCTACTCCATAGCGAATGTTTTTTTAAGCTTGGGCCG