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# Piwi-directed DNA elimination for *Tetrahymena* genetics

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## Abstract

Piwi-bound small RNAs induce programmed DNA elimination in the ciliated protozoan *Tetrahymena*. Using the phenomenon called codeletion, this process can be reprogrammed to induce ectopic DNA elimination at basically any given genomic location. Here, we describe the usage of codeletion for genetic studies in *Tetrahymena* and for investigations of the molecular mechanism of Piwi-directed programmed DNA elimination.

## Key Words

Piwi, small RNA, DNA elimination, gene knockout, *Tetrahymena*

## 1. Introduction

Argonaute proteins are a highly conserved family found in species from humans to archaea and comprise the central protein component of the small RNA-mediated regulatory machinery. Argonaute proteins in eukaryotes can be divided into two subfamilies: the Ago and Piwi subfamilies. While the latter is found predominantly in animals, ciliated protozoans also express Piwi subfamily proteins [1–6]. Therefore, ciliates serve as unicellular models to study the molecular actions of Piwi proteins.

Ciliates are a group of eukaryotes characterized by nuclear dimorphism, in which each cell has a polyploid somatic macronucleus (MAC) and a diploid germline micronucleus (MIC). The former controls metabolism, and the latter functions in reproduction. At each sexual cycle in ciliates, the MIC produces new MICs and new MACs, and the parental MAC is degraded. During the development of the new MAC of *Tetrahymena thermophila*, a model ciliate, ~12,000 DNA segments called internal eliminated sequences (IESs) are reproducibly eliminated [7]. IESs are recognized by 26- to 32-nt small RNAs called scnRNAs [8], which are loaded into the Piwi protein Twi1p [9]. Unlike Piwi-loaded small RNAs in animals, scnRNAs are processed from long double-stranded RNA precursors by a Dicer homolog [10, 11].

IESs in *Tetrahymena* can be classified into two types: Type A IESs, which are recognized in cis by the primary scnRNAs (early scnRNAs) that they produce, and Type B IESs, which are recognized in trans by early scnRNAs produced by Type A IESs [12]. Because secondary scnRNA (late scnRNA) biogenesis occurs concomitantly with the spreading of heterochromatin on IESs [12], the introduction of a chimeric construct of a Type A IES and a fragment of a non-IES locus (targeting sequence) into the new MAC results in the production of secondary scnRNAs from the targeting sequence that induce ectopic DNA elimination at the non-IES locus in trans (Figure 1). This phenomenon is called codeletion (coDel).

Because coDel can be induced by the simple introduction of an IES-targeting DNA chimeric construct, this approach is suitable as a gene knockout technology in *Tetrahymena* [13–19]. Moreover, the effects of an array of different targeting sequences can be easily examined [13], and coDel can also be used to analyze how scnRNAs are involved in the transgenerational inheritance of the pattern of DNA elimination [20] and the base-pairing requirement between small RNAs and genomic DNA to induce the downstream effect. In this article, we describe the

usage of coDel to produce gene knockout strains in *Tetrahymena* and to investigate the length of the seed sequence of scnRNAs in DNA elimination.

## 2. Materials

### 2.1 Extraction of *Tetrahymena* genomic DNA

1. *Tetrahymena thermophila* strain B2086 (or other wild-type strain of *Tetrahymena thermophila*) (Note 1).
2. 1x Super Proteose Peptone (SPP) medium. For the medium recipe, see [22].
3. CHAOS buffer (stored at 4°C): 4 M guanidine thiocyanate, 0.5% sodium lauroyl sarcosinate, 25 mM Tris (pH 8), 0.1 M 2-mercaptoethanol.
4. Phenol extraction buffer (stored at RT): 100 mM Tris (pH 8), 10 mM EDTA (pH 8), and 1% SDS.
5. Phenol-chloroform-isoamyl alcohol=25:24:1, saturated with 10 mM Tris (pH 8) and 1 mM EDTA (Sigma P2069)
6. 2-propanol.
7. 70% ethanol.
8. Ethanol.
9. TE (Tris-EDTA) buffer (pH 8).
10. RNase A (10 mg/mL, Thermo Fisher Scientific EN0531).

### 2.2. Construction of coDel-targeting plasmid

1. pMcoDel plasmid (Note 1).
2. FW and RV primers amplifying the ~500-1000 bp region of a targeted locus (Note 2). To assemble a construct using the NEBuilder HiFi DNA Assembly system, the primers must have >16 nt overlapping sequence with pMcoDel arms (Note 3). For example, to induce deletion at the *TTHERM\_00113330* locus (Figure 2A), we used EMA2-coDel-FW and EMA2-coDel-RV (Table 1). To assemble a construct using T4 DNA ligase, both primers must have the NotI recognition site (Note 4). For example, to induce deletion at a noncoding sequence on MIC chromosome 4 with the 661 bp targeting sequence (Figure 3A), we used C121\_FW1 and C121\_RV1 (Table 1).
3. Taq DNA Polymerase with Standard Taq Buffer (NEB M0273).
4. 2.5 mM each dNTP mix.
5. NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel 740611).
6. NotI-HF (NEB).
7. NEBuilder HiFi DNA Assembly Master Mix (NEB E2621).
8. FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific EF0651).
9. DNA Ligation Kit, Mighty Mix (Takara 6023).
10. NEB 5-alpha Competent *E. coli* (NEB C29871): thaw on ice, aliquot 12.5 µL each in screw-capped 1.5 mL tubes, freeze with liquid nitrogen, and store at -80°C.
11. Primers for colony PCR and sequencing (McoDel\_seqFW and McoDel\_seqRV [Table 1]).

### 2.3. Transformation for inducing coDel

1. Two wild-type *Tetrahymena thermophila* strains having complementary mating types. We typically use B2086 (Mating Type II) and CU428 (Mating Type IIV) (Note 1).
2. Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). (Note 5) (Note 6).
3. 10 mM Tris pH 7.5.

4. 1x and 2x Super Proteose Peptone (SPP) medium. [22].
5. 100 mg/mL paromomycin sulfate (Sigma Aldrich P8692-25G or Toronto Research Chemicals P205650).
6. 100x Antibiotic/Antimycotic (AB/AM) mix (Millipore, Cnt-ABT).
7. 96-well cell culture plates.
8. 30°C Incubator.

#### 2.4. Detecting coDel by direct PCR

1. Primers amplifying the targeted region and its ~1 kb flanking sequences. To examine the *THERM\_00113330* locus, we used either EMA2\_coDel\_SeqFW1 and EMA2\_coDel\_SeqRV (primers #1 and #3 in Figure 2A) or EMA2\_coDel\_SeqFW2 and EMA2\_coDel\_SeqRV (primers #2 and #3 in Figure 2A).
2. GoTaq Long PCR Master Mix (Promega, M4021).

#### 2.5. Establishing gene knockout cell lines from cells with coDel

1. DropMaker (made-to-order, see [22] for more explanations).
2. Bacteriological petri dish (100 x 15 mm, Falcon 351029)
3. Glass capillary (Kimble 34502-99)
4. Aspirator tube assemblies for microcapillary pipettes (Sigma A5177-5EA)
5. Glass capillary puller (Narishige PC-100)
6. Dissection Microscope.
7. 24-well and 96-well cell culture plates.
8. 13.3% DMSO in 10 mM Tris (pH 7.5) (Note 7).
9. Cryo 1°C “Mr. Frosty” Freezing Container (Nalgene 5100-0001).

#### 2.6. Using coDel to study DNA elimination mechanism

1. Primers to amplify different regions of target sequences (Figure 3A) (Table 1).
2. Synthetic double-stranded DNA with base replacements at defined intervals (Integrated DNA Technologies) (Note 8).
3. Synthetic single-stranded DNA corresponding to a part of the target sequence and having base replacements at defined intervals (such as Ultramer DNA oligos from Integrated DNA Technologies) (Table 1).
4. Primers amplifying the targeted region and its ~1 kb flanking sequences (Table 1).

### **3. Methods**

#### 3.1 Extraction of *Tetrahymena* genomic DNA

1. Culture the B2086 strain of *Tetrahymena* cells in 20 mL 1x SPP at 30°C overnight (Note 9).
2. Place approximately 1 mL of the culture (~1 x 10<sup>6</sup> cells) in a 1.5 mL Eppendorf tube, centrifuge for ~5 sec in a mini table top centrifuge, and quickly remove the supernatant using a pipette.
4. Add 300 µL CHAOS buffer and lyse cells by pipetting (Note 10).
5. Add 300 µL of Phenol Extraction Buffer and mix vigorously by inverting the tube (Note 11).
6. Add 600 µL of phenol/chloroform/isoamyl alcohol and mix vigorously by inverting the tube.
7. Spin down for 10 min at 14,000 rpm (or maximum speed) at RT and transfer 600 µL of the upper phase to a fresh 1.5 mL tube.
8. Add 600 µL of 2-propanol and mix well by inverting the tube.

9. Spin down for 10 min at 14,000 rpm at 4°C and discard supernatant.
10. Add 700 µL ice-cold 70% ethanol, leave for 5 min at RT and spin down for 5 min at 14,000 rpm at 4°C.
11. Discard the supernatant as much as possible and air dry for 5-10 min.
12. Add 200 µL TE buffer and incubate at RT for ~15 min (vortex occasionally) ([Note 12](#)).
13. Add 1 µL of RNaseA and incubate at 37°C for 30 min.
14. Add 200 µL TE buffer and 400 µL phenol/chloroform/isoamyl alcohol, mix by inverting tube several times, spin down for 10 min at 14,000 rpm at RT.
15. Transfer the upper phase to a fresh 1.5 mL tube, add 5% vol. of 5 M NaCl and mix well.
16. Add 2x vol. of ethanol, mix well and incubate the tube at -20°C for more than 1 h.
17. Spin down for 10 min at 14,000 rpm at 4°C and discard the supernatant.
18. Add 700 µL ice-cold 70% ethanol and spin down for 5 min at 14,000 rpm at 4°C.
19. Remove supernatant as much as possible and air dry for 5-10 min.
20. Add 100 µL TE buffer and incubate for ~15 min at RT (vortex occasionally).
21. Measure the DNA concentration, adjust to 0.5 µg/µL by adding TE and store at -20°C.

### 3.2 Construction of coDel-targeting plasmid

1. Set up a 50 µL PCR containing 1x PCR buffer (NEB), 100 µM each dNTP, 0.2 µM FW primer, 0.2 µM RV primer, 10 ng Tetrahymena genomic DNA (3.2.24), and 1.25 Unit Taq polymerase.
2. Perform PCR with the following conditions: 95°C-2 min, 35 cycles of 95°C-20 sec/55°C-30 sec/68°C-60 sec, 68°C-3 min
3. Check the product by running 3 µL of the reaction in a 1% agarose gel.
4. Purify the PCR product using NucleoSpin Gel and PCR Clean-up Kit (or similar glass filter base purification system). If necessary, run the PCR product in a 1% agarose gel and excise the correct PCR product.
5. Digest ~1-2 µg of pMcoDel plasmid DNA with NotI-HF and 1xCutSmart buffer in 50 µL overnight. If the NEBuilder HiFi DNA Assembly system will be used, go to Step 6. When T4 DNA ligase will be used, go to Step 9.
6. Extract NotI-digested pMcoDel with NucleoSpin Gel and PCR Clean-up Kit and elute with 30 µL elution buffer.
7. Mix 1 µL of NotI-digested pMcoDel (Step 6), 1 µL PCR-amplified targeting sequence (Step 4) and 2 µL of 2x NEBuilder HiFi DNA Assembly Mix
8. Incubate at 50°C for 30 min and go to Step 14.
9. Add 1 µL of FastAP, incubate at 37°C for 1 h and then inactivate the enzyme for 15 min at 65°C.
10. Extract DNA with a NucleoSpin Gel and PCR Clean-up Kit and elute with 30 µL elution buffer.
11. Digest the PCR product (Step 4) with NotI-HF as in Step 5; follow by purification using a NucleoSpin Gel and PCR Clean-up Kit.
12. Mix 1 µL of NotI-digested and dephosphorylated pMcoDel (Step 10), 1 µL NotI-digested PCR product (Step 10) and 2 µL of 2x ligation mix.
13. Incubate at 16°C for a few hours to overnight.
14. Transform NEB5alpha competent cell (12. 5 µL) with a 0.5 µL reaction (either from Step 8 or 13) and inoculate on an LB-amp plate.
15. Check the resulting plasmid construct by colony PCR using McoDel\_seqFW and McoDel\_seqRV.
16. Purify the plasmid construct with the correct insert size by mini-prep, and check the sequence of the insert by Sanger sequencing using McoDel\_seqFW.

### 3.3. Transformation for inducing coDel

1. Mix 25 mL each  $5 \times 10^5$ /mL prestarved B2086 and CU428 in 10 mM Tris pH 7.5 ([Note 9](#)).
2. Introduce 5  $\mu$ g of pMcoDel-Targeting Sequence plasmid into  $6 \times 10^6$  cells at 7 hpm using BioRad Biolistic Particle Gun system ([Note 5](#)).
3. Release cells into 50 mL 10 mM Tris-HCl (pH 7.5) and incubate for  $\sim 12$  h at 30°C with slow (60 rpm) rotation.
4. Add 50 mL of 2xSPP and 500  $\mu$ L of 100x AB/AM mix.
5. Incubate at 30°C for an additional  $\sim 4$  h with slow (90 rpm) rotation.
6. Add 100  $\mu$ L of 100 mg/mL paromomycin (final conc. 100  $\mu$ g/mL).
7. Make two diluted cultures: a) 1.5 mL of the culture + 13.5 mL of 1x SPP + 135  $\mu$ L 100x AB/AM mix + 13.5  $\mu$ L of 100 mg/mL paromomycin; b) 0.3 mL of the culture + 14.7 mL of 1x SPP + 100x AB/AM mix + 14.7  $\mu$ L of 100 mg/mL paromomycin.
8. Aliquot cells into 96-well plates.
  - Original culture: 150  $\mu$ L/well, 7x 96-well plates
  - 1/10 diluted culture (5-a): 150  $\mu$ L/well, 1x 96-well plates
  - 1/50 diluted culture (5-b): 150  $\mu$ L/well, 1x 96-well plates
9. Place the plates in a moist chamber (plastic box with wet tissue papers) and incubate for 3-4 days at 30°C ([Note 13](#)).
10. Select 24 wells containing paromomycin resistance cells from 96-well plate(s) in which 2/3 or less of the wells had growing cells ([Note 14](#)).

### 3.4. Detecting coDel by direct PCR

1. Mix the following in a PCR tube and keep on PCR cooler (or on ice): 10  $\mu$ L GoTaq Long PCR Master Mix (2x), 0.5  $\mu$ L 10  $\mu$ L FW primer, 0.5  $\mu$ L 10  $\mu$ L RV primer, 8.5  $\mu$ L water.
2. Add 0.5  $\mu$ L culture (3.2.8) to the PCR tube above (3.3.1).
3. Perform PCR under the following conditions: 94°C for 2 min; 35 cycles of 94°C for 20 sec, 50-55°C for 30 sec, and 67°C for 1 min per kb; then 67°C for 5 min.
4. Analyze 3-5  $\mu$ L of reaction by agarose gel electrophoresis (Figure 2B as an example).

### 3.5. Establishing gene knockout cell lines from cells with coDel

1. Select 6 to 8 cell lines in which deleted copies of the target locus are dominant over the wild-type copy in 3.4.4 ([Note 15](#)).
2. Prepare 48 drops of 1x SPP on a 10 cm culture dish (1 plate for 2 lines) using a DropMaker. Alternatively, drops ( $\sim 50$   $\mu$ L each) can be made using a pipet.
3. Pick up 24 single cells each from the corresponding culture (3.2.10) using a microcapillary pipette and separately placed into the drops above.
4. Incubate for 3 days at 30°C in a moist chamber.
5. Choose 6-8 drops containing well growing cells per line and transfer them individually into 1 mL fresh 1x SPP in a 24-well plate.
6. Incubate for 2 days at 30°C.
7. Perform direct PCR as in 3.4 to find clones showing only deleted copies of the target locus (Figure 2C as an example) ([Note 16, 17](#)).
8. (If sexually mature cells are needed for the experiment) Every two days, inoculate 5  $\mu$ L of the selected clones into 1 mL fresh 1x SPP and incubate at 30°C (or keep cells at room temperature for a week) until passage 10. Then, perform test mating to identify combinations of clones with complementary mating types.

9. Prepare frozen cell stocks using DMSO and Cryo 1°C Freezing Container. Refer to [22] for detailed cell storage protocol.

### 3.6. Using coDel to study DNA elimination mechanism

1. Design modified targeting sequences (Figure 3A as examples) (Note 18) and prepare them by PCR from the original target or by gene synthesis.
2. Introduce the modified target sequences into pMcoDel as in 3.2.4-3.2.16.
3. Introduce the resulting constructs into mating *Tetrahymena* cells as described in 3.3.
4. Analyze the target locus by direct PCR as in 3.4.
5. Measure the deletion frequency by counting the number of cell lines showing PCR products shorter than that from the wild-type target locus (Figure 3A) (Note 19).

### **4. Notes**

1. The wild-type *Tetrahymena thermophila* strains and pMcoDel plasmid are available from the *Tetrahymena* Stock Center <<http://tetrahymena.vet.cornell.edu/>>.

2. Our previous study suggested that coDel can be induced if an ~1 kbp segment shares ~ 90% identity with the targeting sequence [13]. The results shown in Figure 3 also support this view. We therefore suggest choosing a target sequence that does not share >90% identity with any other genomic locations in all possible 200 bp windows in the genome. Nonetheless, off-target deletion may be induced in an unpredictable manner even with a carefully chosen target. Therefore, it is important to perform a genetic rescue experiment to confirm whether an observed phenotype is caused by the deletion at the targeted locus but not by some off-target deletion.

3. These designs work well for assembly by the NEBuilder HiFi assembly system: FW = 5'-CTTTATTGTTATCATCTTATGACCGC-target-3'; RV = 5'-CTCATCAAGTTGTAATGCTAAAATGC-target-3'.

4. 5'-GCATGCGGCCGC-target-3' for both primers works fine for ligation.

5. For preparation of materials and detailed protocols for biolistic transformation, refer to [23]

6. Because pMcoDel is based on the high-copy rDNA vector pD5H8 [24], a targeting construct can also be introduced into the new MAC by electroporation [25]. However, we never tested whether coDel could be induced by electroporation of any construct.

7. Aliquots of 1.33 mL of DMSO from freshly opened bottles were placed into 15 mL tubes and stored at -20°C. Add 8.67 mL 10 mM Tris (pH 7.5) immediately before preparation of frozen cell stock.

8. The AT-rich genome of *Tetrahymena* sometimes prevents efficient delivery of synthetic double-stranded DNA corresponding to some *Tetrahymena* genome loci. If an alternative region cannot be chosen, multiple shorter synthetic single-stranded DNAs can be stitched by overlapping PCR to produce the desired DNA fragment).

9. For detailed *Tetrahymena* cell culture and mating conditions, see [22] and [26].
10. Cells lysed in CHAOS can be stored at -20°C for more than several months.
11. Solution will be opaque. If not, add ~30  $\mu$ L more phenol extraction buffer.
12. Steps 3.1.13 to 3.1.21 are not necessary to amplify up to ~1 kb DNA from the MAC by standard Taq polymerases. These steps enhance amplification of longer DNA from the MAC or any size of the MIC DNA by Taq polymerase. These steps are often necessary to amplify DNA from any genomic region by high-fidelity polymerases such as Q5 DNA polymerase (NEB) or PrimeSTAR HS DNA Polymerase (Takara).
13. pMcoDel is derived from the rDNA vector pD5H8, which contains the MIC copy of the rDNA locus containing a point mutation that causes the derived rRNA to be insensitive to paromomycin [24]. Because mature, functional rDNA can be produced from the rDNA vector only when it undergoes programmed genome rearrangement in the developing new MAC, all paromomycin-resistant cells obtained by introduction of pMcoDel should be sexual progeny of B2086 and CU428 and have pMcoDel-derived mature rDNA in the (new) MAC.
14. It is preferable to avoid plates in which all wells have growing cells because, in such a plate, the cells in each well were likely established from multiple transformation events.
15. The efficiency of coDel differs among targets. If more than 6 cell lines with appropriate deletions at the targeted locus are not obtained, it is necessary to screen more transformants, target different regions of the targeted gene, and/or use a longer targeting sequence. In our experience, extending the targeting sequence longer than 1 kb does not obviously enhance coDel.
16. If the wild-type locus is not detected in the selected line before cloning in section 3.3.4, examining a few clones is probably sufficient to obtain a clone in which all the copies of the target locus in the polyploid MAC are deleted.
17. Because coDel induces DNA elimination only in the MAC, genomic PCR may detect the intact wild-type (nondeleted) copy of the targeted locus in the MIC even when all the copies in the MAC have deletions. Therefore, complete absence of the wild-type copy of the targeted gene in the MAC in selected strains may be necessary to be confirmed at the product level (such as by western blot, RT-PCR or northern blot).
18. A series of truncated sequences and sequences having base replacements in defined intervals.
19. When the 401 bp targeting sequence has base replacement mutations every 10 bp and scnRNAs use X nucleotides as seeds to initiate recognition of the targeted sequences, the total length of the targetable sequence by such scnRNAs (Y) can be estimated as  $Y = 401 - (x-1) \cdot 40x$ . From the truncation study above, the minimum length of the targeting sequence to induce some DNA elimination at this targeted locus was ~150 bp (Figure 3B). Because the targetable sequence length (Y) becomes ~150 bp when  $X = 6$  (Figure 3C), we can estimate that the scnRNAs use more than 6 nucleotides for their seed sequence. This estimation of the seed



length also explains the result obtained by the 401 bp targeting sequence with mutations every 20 bp, which is estimated to reduce the actual targetable sequence length to 276 bp if the seed size is 6 nt (Figure 3C). A 276 bp targeting sequence is predicted to induce coDel in 15.3% of transformants (Figure 3B), which was close to the actual fraction of transformants with deletion by this targeting sequence (=21.7%, Figure 3A). The seed of scnRNA in Twi1p-scnRNA complex during *Tetrahymena* DNA elimination is most likely 6-8 bases, as in other Argonaute-mediated systems, and some mismatch(s) in the seed can be tolerated if other regions of the scnRNA are also complementary to the target.

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## Figure Legends

### Figure 1. The proposed actions of scnRNAs in coDel.

(A) DNA elimination without induction of coDel. Early scnRNAs (magenta wavy lines) produced from Type-A IESs (represented by M-IES in this figure) in the MIC (i) interact with Type-A IESs in the new MAC and induce DNA elimination (ii). Non-IES loci (MAC-destined sequences [MDSs]) either do not produce early scnRNAs or homology-dependent small RNA degradation eliminates early scnRNAs derived from them [8, 12, 20, 21]; thus, DNA elimination is not induced at these loci. (B) Induction of coDel. When an IES (magenta box)-targeting sequence (green box) chimeric construct (pMcoDel+Targeting Seq) is introduced into the new MAC, early scnRNAs produced from M-IES in the MIC (i) interact not only with the endogenous M-

IES (ii) but also with the M-IES on the introduced chimeric construct (iii). The latter interaction triggers late scnRNA (green wavy lines) production from the adjacent target sequence in cis (iv), which then interacts with the endogenous target locus in trans (v) and induces ectopic DNA elimination (vi). Late scnRNAs likely further induce the production of late scnRNAs in cis at the targeted locus (vii), making deletions longer than the targeted sequence.

### **Figure 2. Production of Gene Knockout strains using coDel**

(A) A coDel was induced at the coding sequence of *TTHERM\_00113330* (blue boxed arrow) in the MAC by inserting an ~1 kb sequence at the beginning of the *TTHERM\_00113330* coding sequence (red box) into the pMcoDel vector.

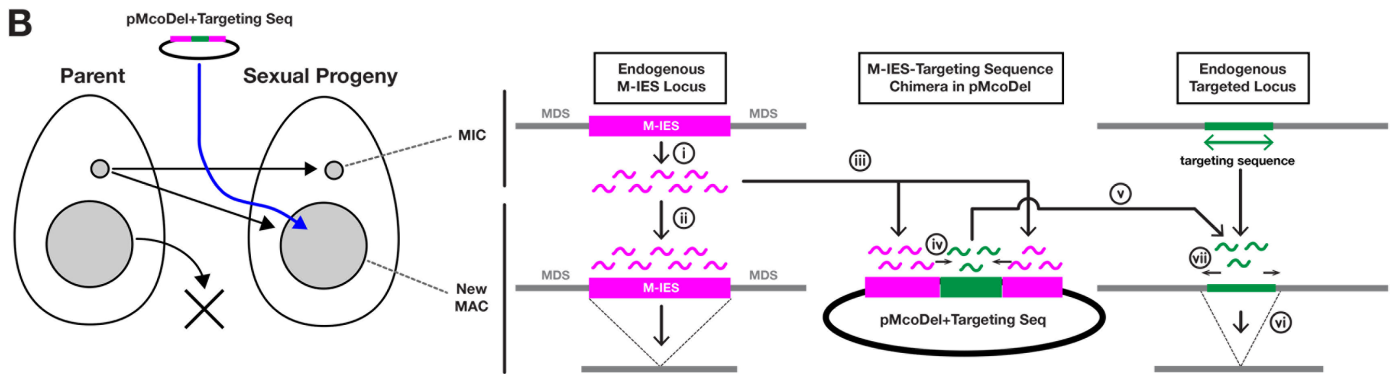
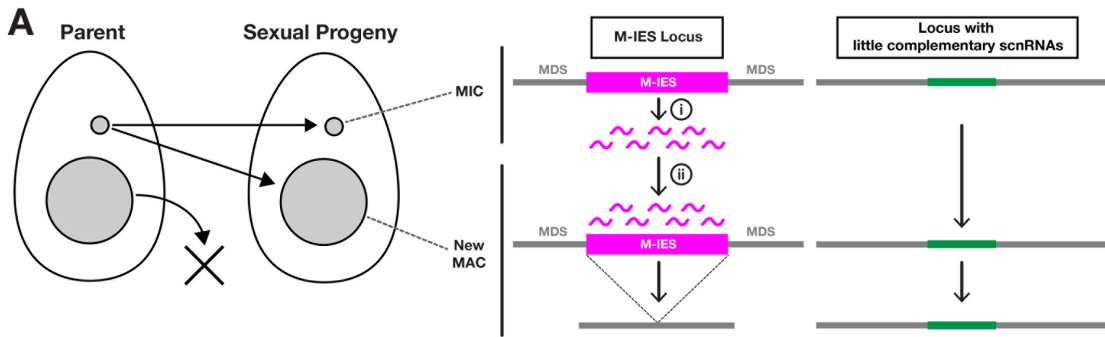
(B) Wild-type (WT) and 23 paromomycin-resistant transformants after introduction of the pMcoDel-*TTHERM\_00113330* targeting construct (deletion induced) were examined by direct cell PCR using primers #1 and #3 shown in (A). The sequences of the primers are listed in Table 1. A 2,625 bp product was expected to be obtained without any deletion at the targeted locus (as in WT), and all of the coDel-induced cell lines showed ~1.2 to 2 kb PCR products, which correspond to the locus with a deletion at the targeted region.

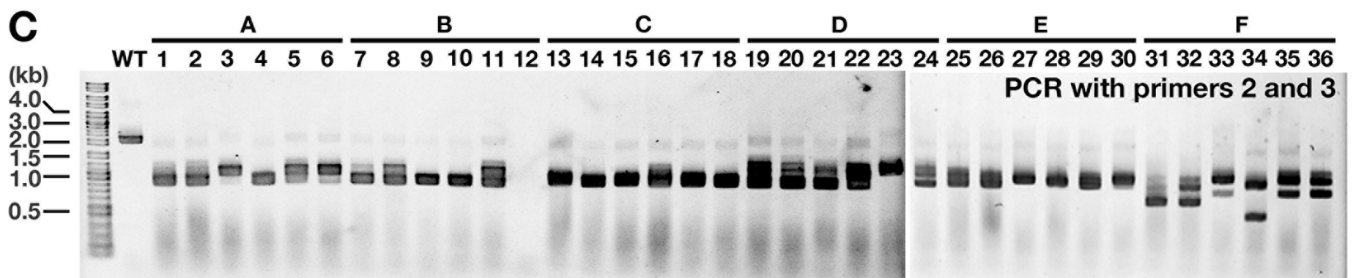
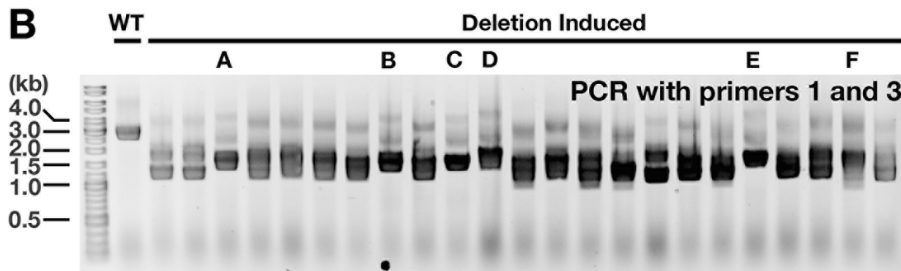
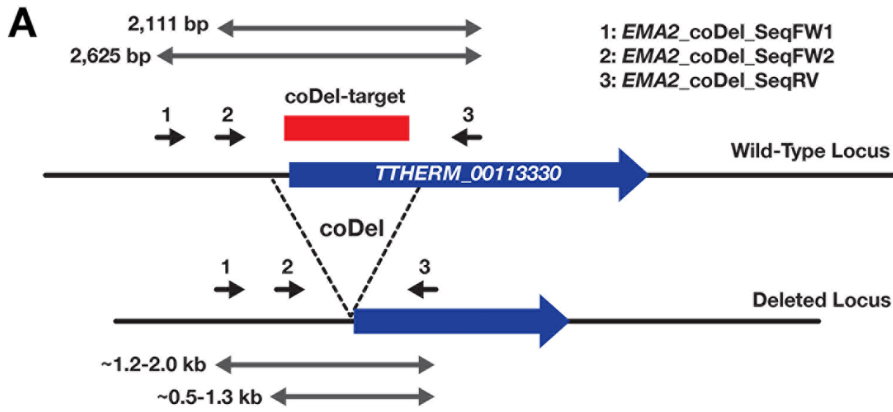
(C) Six coDel lines (marked with A to F in (B)) were selected, six clonal cell lines each were established, and their *TTHERM\_00113330* locus was analyzed by PCR using primers #2 and #3 shown in (A). The sequences of the primers are listed in Table 1. A 2,111 bp product was expected to be obtained without any deletion at the targeted locus (as in WT), and all of the coDel-induced cell lines showed ~0.5 to 1.2 kb PCR products.

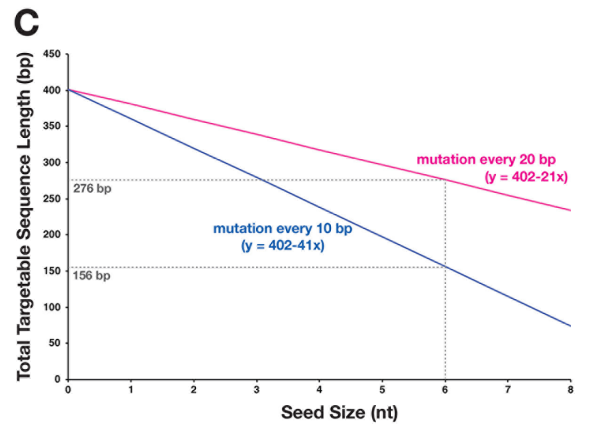
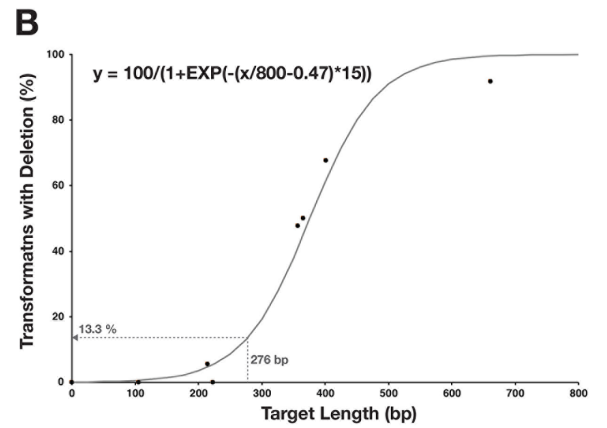
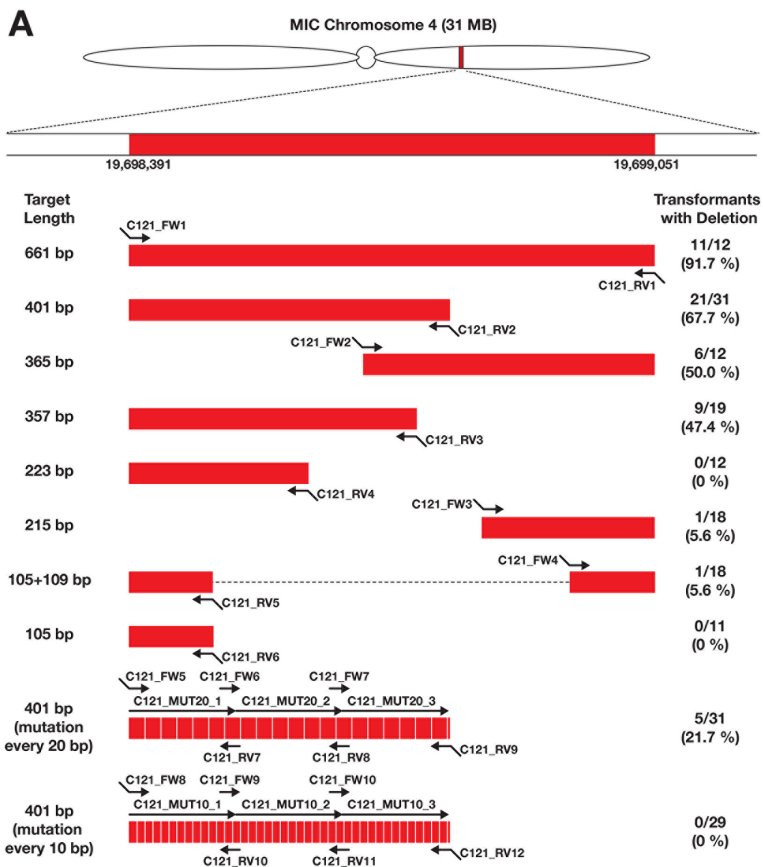
### **Figure 3. Examination of the requirement of scnRNA-target interaction in DNA elimination using coDel.**

(A) Various targeting sequences that are designed to target a non-IES region at the indicated position of MIC chromosome 4 (red boxes) were introduced into the new MAC of progeny of B2086 and CU428, and the number of transformants that showed deletion at the targeted locus was counted. Arrows indicate the primers and oligo DNAs (their sequences are listed in Table 1) that were used to amplify and construct the targeting sequences.

(B) Length of the different targeting sequences shown in (A) (except the last two having base replacements) and fraction of transformants that showed deletion by those targeting sequences are plotted. A sigmoidal curve that is close to the plotted experimental data is shown. Dotted lines indicate the expected fraction of deletion-positive transformants by a 276 bp targeting sequence, which corresponds to the total targetable sequence lengths if scnRNAs from the 401 bp targeting sequence having base replacement every 20 nt use 6 nt as seed (see below). (C) Total targetable sequence lengths within the 401 bp targeted sequence by the scnRNAs derived from the 401 bp targeting sequence that have base replacement in every 20 (magenta line) or 10 (blue line) bp were predicted (y-axis) for different seed sizes to initiate recognition of the targeted sequences by scnRNAs (x-axis). Dotted lines indicate length of predicted total targetable sequence when scnRNA uses 6 nt as seed. See also Note 13 for interpretation of the results.







**Table 1 Sequences of Synthetic DNA**

<b>Primer Name</b>	<b>Sequence (5'–3')</b>
EMA2_coDel_FW	<u>ATTGTTATCATCTTATGACCGCTTTAATGTATTTGACTAGTGC</u>
EMA2_coDel_RV	<u>TCAAGTTGTAATGCTAAAATAAGCAAATGAGTTCACACTATTTGG</u>
EMA2_coDel_SeqFW1	AATTGGGTACCGGGCCCCCCTCGAGGTGGATCTGCTGGAAAAAGATTGTATG
EMA2_coDel_SeqFW2	GCTGGCGGAGATATGCAAGATGGTC
EMA2_coDel_SeqRV	GACTAAGTCCTCTATTGGGTTTGTTC
McoDel_seqFW	ATTGAATAAGGAGACCAGCCTCTC
McoDel_seqRV	AAAAC TAATAACTTGGGTCTTTAGATCAC
C121_coDel_cFW	GGTTGATGGTAGGTAGGTACCTC
C121_coDel_cRV	CAGTAGATTTCTTTGAAAGCTAACTC
C121_FW1	<u>GCATGCGGCCGCGAGACATTTCTGGTGTGTTCCG</u>
C121_FW2	<u>GCATGCGGCCGCGAGAGTTAATCTTTATTTAAAAGGC</u>
C121_FW3	<u>GCATGCGGCCGCGTATTTTTAGTAACATGTATCAAG</u>
C121_FW4	CAAAGTTGATGTTTTCTGGTGGTTACAGTTGATAAAAAATTAG
C121_FW5	<u>GCATGCGGCCGCGAGACATTTCTGGTGTGTTGG</u>
C121_FW6	TAAGTACGTCATAATATATTGTTTTCTTGA
C121_FW7	TGACTAGTTTGTAAATTACTAAATTTAA
C121_FW8	<u>GCATGCGGCCGCGAGACATTTGTGGTGTGTTGG</u>
C121_FW9	TAAGTACGTCATAATATATTGTTTTCTTGA
C121_FW10	TGACTAGTTTGTAAATTTTACTAAATTTAA
C121_RV1	<u>GCATGCGGCCGCGATTAATTGGCTATTCAAGCTATG</u>
C121_RV2	<u>GCATGCGGCCGCTTGGTGAGGAACCAAATATGTTTG</u>
C121_RV3	<u>GCATGCGGCCGCTTCATGGGGAGTTAATAATTATC</u>
C121_RV4	<u>GCATGCGGCCGCCCCAACAAATTAGATATAATTTCCC</u>
C121_RV5	CTAATTTTTTATCAACTGTAACCACCAGAAACATCAACTTTG
C121_RV6	<u>GCATGCGGCCGCGCACCAGAAACATCAACTTTG</u>
C121_RV7	TCAAGAAAACAATATATTATGACGTACTTA
C121_RV8	TTAAATTTAGTAATAATTACAACTAGTCA
C121_RV9	<u>GCATGCGGCCGCTAGGTGAGGAACCAAATATGTATG</u>
C121_RV10	TCAAGAAAACAATATATTAAGACGTACTTA
C121_RV11	TTAAATTTAGTAAAAATTACAACTAGTCA
C121_RV12	<u>GCATGCGGCCGCTAGGTGAGGAAGCAAATATGTATG</u>
C121_MUT20_1	GAGACATTTCTGGTGTGTTGGGAATTCTATTTAAAACCTTTCTATGTAATCTAACT TTTTATTTTTTATAAAAAATTTAAAATAAAAAACAAAGTTGATGTTTGTGGTGATA ATTCATAAATATAAGTACGTCAATAAT
C121_MUT20_2	ATATTGTTTTCTTGAATATAAGTATAAACTAATTAAGTTAAGTAACTAAATAAG GTGAAGGATTTCCGAAATTATATCTAATTGTAGGGTAAATTTATAATTTTCTACT CTTTTTTATCTTGACTAGTTTGTAAT
C121_MUT20_3	TATTACTAAATTTAATTCAATTTATTGAGAGTGTTAATCTTTATTTAAAAGGGTC AGAAAATAATAGATAATAATTAACCTCCCATGAAGATTAGATAGAAACAAACAA ACATACATATTTGGTTCCTCACCTA
C121_MUT10_1	GAGACATTTGTGGTGTGTTGGGAATTCTAATAAAAACCTTTCTATGTAATCAAACCT TTTTATTTTTTATAATAATTTAAAATAAAAAACAACTTGTATGTTTGTGGTGATA AATCATAAATATAAGTACGTCTTAAT
C121_MUT10_2	ATATTGTTTTCTTGATTATAAGTATAAACTAATTATGTTAAGTAACTAAATAAG GAGAAGGATTTCCGAAATTATTTCTAATTGTAGGGTAAATTTTAAATTTTCTACT CTTTTTTTCTTGACTAGTTTGTAAT
C121_MUT10_3	TTTTACTAAATTTAATTCAATATATTGAGAGTGTTAATCTTAATTTAAAAGGGTC AGAAAATTATAGATAATAATTAACCTCCGCATGAAGATTAGATAGAAAGAAACAA ACATACATATTTGCTTCCTCACCTA

Underlined and double-underlined sequences are for NotI digestion and NEBuilder HiFi assembly to the arms of pMcoDel, respectively.