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Rapid generation of maternal mutants via oocyte transgenic expression of CRISPR-Cas9 and sgRNAs in zebrafish

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1 **FRONT MATTER**

2

3 **Title**

- 4 • Rapid generation of maternal mutants via oocyte transgenic expression of CRISPR/Cas9
5 and sgRNAs in zebrafish
6 • Maternal mutants generated by multiple Cas9 RNPs
7

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Abstract

Maternal products are exclusive factors to drive oogenesis and early embryonic development. As disruption of maternal gene functions is either time-consuming or technically challenging, early developmental programs regulated by maternal factors remain mostly elusive. This study provides a transgenic approach to inactivate maternal gene functions in the zebrafish primary oocyte. By introducing three tandem sgRNA expression cassettes and a GFP reporter into Tg(*zpc:zcas9*) embryos, we efficiently obtained maternal *nanog* and *ctnnb2* mutants among GFP-positive F1 offspring. Notably, most of these maternal mutants displayed either sgRNA site-spanning genomic deletions or unintended large deletions extending distantly from the sgRNA targets, suggesting a prominent deletion-prone tendency of genome editing in the oocyte. Thus, our method allows maternal gene knockout in the absence of viable and fertile homozygous adults. This approach is particularly time-saving and technically accessible. It can be applied for functional screening of maternal factors and generating genomic deletions in zebrafish.

Teaser

A time-saving and deletion-prone method to inactivate maternal factors in zebrafish.

40 **MAIN TEXT**

41

42 **Introduction**

43 Maternally supplied mRNAs and proteins, produced by over half of the coding genes in
44 zebrafish (*1*), control oogenesis and the earliest processes of embryonic development, especially
45 those before zygotic genome activation (*2-6*). Some maternal factors can also perform zygotic
46 functions during early development (*7-9*). Thus, eliminating both maternal and zygotic products is
47 essential to elucidate functional roles of genes with maternal expression. However, most maternal
48 gene products are cell-autonomously deposited in primary oocytes that have the same genotype as
49 somatic cells. As a result, to generate maternal (M) or maternal-zygotic (MZ) mutant offspring by
50 traditional genetic approach, viable and fertile homozygous mutant females are indispensable.
51 These homozygous mutants are classically produced in a time-consuming manner by repeated
52 crossing and screening through typically three generations (almost nine months in zebrafish).
53 Nevertheless, if loss of zygotic gene functions causes death or sterility, it becomes particularly
54 difficult to produce maternal mutants. One way to circumvent these obstacles is to rescue
55 phenotype defects by injecting the wild-type mRNA, such as the case for the generation of *Moep*
56 mutants (*8*), but this approach was proved to be possible only for a small proportion of zygotic
57 lethal genes. Several alternatives have also been developed, such as germ-line replacement (*9-12*),
58 oocyte microinjection in situ (*13*), generation of genetically mosaic females (*7*), or the BACK
59 approach using a BAC plasmid containing the wild-type gene that is sent "BACK" to the genome
60 for rescue (*14*). These methods remain technically challenging, time-consuming, or less efficient.
61 Therefore, a rapid and straightforward system to inactivate genes in the oocyte may break the
62 barrier to study their maternal functions.

63 The CRISPR/Cas9 system has become a powerful tool in genome editing, promoting
64 extensive functional analyses (*15-17*). CRISPR/Cas9-based conditional knockout has been tested
65 in several species, including zebrafish, mouse and mosquito (*18-21*). The strategy to achieve
66 tissue-specific or temporally controlled gene inactivation relies on the transgenesis and the use of
67 promoters to drive the spatiotemporal expression of Cas9 protein (*18-28*). However, the
68 application of Cas9-mediated conditional knockout is still limited, probably due to the low
69 efficiency and mosaicism or failure to ensure complete bi-allelic mutations (*18, 19, 25, 29-31*).
70 These often result in incomplete removal of tissue-specific gene products or functional
71 compensation from wild-type or heterozygous cells, thus generating animals without detectable

72 mutant phenotypes. Distinct from tissues or organs in which component cells function as a whole,
73 every oocyte with bi-allelic maternal gene disruption in the ovary can lead to the generation of a
74 homogeneous maternal mutant animal. Therefore, we reasoned that CRISPR/Cas9-mediated
75 oocyte-specific conditional knockout should still be highly applicable to create maternal mutants,
76 despite the moderate efficiency and mosaic feature of the CRISPR/Cas9 system in transgenesis.

77 Zona pellucida gene *zp3b* (also known as *zpc*) is specifically expressed at early stages of
78 oocytes, and its promoter can drive a high level of maternal expression of GFP (32). We have
79 previously created a transgenic line Tg(*zpc:zcas9*) that specifically expresses zebrafish codon-
80 optimized *cas9* in the oocytes, and tested its application in routine gene inactivation by injection
81 of sgRNAs into the embryo (33, 34). In this study, we developed a rapid conditional knockout
82 strategy to generate maternal mutants by just one generation (less than three months). By
83 introducing a single or multiple sgRNA expression modules into Tg(*zpc:zcas9*) embryos via I-Sce
84 I-mediated transgenesis, we successfully generated maternal mutants for *nanog* and *ctnnb2* (β -
85 *catenin2*) genes among F1 embryos. We also discovered a dramatic tendency for genome editing
86 in zebrafish oocytes to frequently produce heritable large deletions when expressing multiple
87 sgRNAs. Therefore, our study demonstrates the promising potential of this novel conditional
88 knockout strategy to circumvent current technical restrictions in functional studies of maternal
89 factors.

90

90

91 **Results**

92 **Experimental design of a rapid oocyte-specific conditional knockout system**

93 As Tg(*zpc:zcas9*) zebrafish has adequate maternal Cas9 protein for genome editing in the early
94 embryos (34), we were wondering if it was possible to directly inactivate genes of interest in the
95 oocytes by introducing a transgenic sgRNA expression cassette in order to produce maternal
96 mutants by just one generation. We thus designed a transgenic vector pGGDestI-SceIEG-1sgRNA
97 containing the U6a promoter to drive the expression of a single sgRNA and an *ef1a* promoter to
98 drive the ubiquitous expression of GFP that will serve as a selection marker. These two modules
99 are flanked by Tol2 inverse repeat sequences (TIR) and I-Sce I restriction sites (Fig. 1A). By
00 Golden Gate ligation, we can also connect three sgRNA expression modules driven by U6a or
01 U6b promoter to create a pGGDestI-SceIEG-3sgRNA vector (Fig. 1B). This endows the vector
02 with the ability to express three sgRNAs simultaneously targeting a single gene of interest,
03 potentially improving the efficiency of bi-allelic gene disruption (35).

04 Because Tg(*zpc:zcas9*) zebrafish line was created by Tol2 transposition, we decided to use
05 meganuclease-mediated transgenesis to integrate the sgRNA expression cassette into the genome
06 (36), such that an accidental removal of the *zpc:zcas9* transgene could be avoided. Embryos from
07 a wild-type female and a homozygous Tg(*zpc:zcas9*) male are employed for further I-Sce I-
08 mediated transgenesis. After coinjection of I-Sce I and sgRNA transgenic vector, mosaic embryos
09 with wide-spread GFP expression were selected and raised to adulthood. The mosaic F0 female
10 fish will be subsequently outcrossed with wild-type males to check sgRNA expression in the
11 embryos by monitoring the expression of maternal GFP. Finally, maternal mutants will be
12 identified by phenotyping and genotyping GFP-positive offspring (Fig. 1C).

13

14 **A proof-of-principle to produce maternal *nanog* mutants in F1 embryos**

15 As a proof-of-principle, we selected *nanog* gene as a target because its maternal products are
16 required for zygotic transcription of many essential genes involved in pattern formation and cell
17 movements during gastrulation (37-39). As a result, maternal *nanog* mutant (*Mnanog*) embryos
18 can be readily identified from 5 hours post-fertilization (hpf) onward by the extremely delayed
19 epiboly phenotype, whereas zygotic homozygous mutant embryos develop normally. An sgRNA
20 target was selected in the first exon of *nanog* gene by CRISPRscan (40) and was found to be

21 highly efficient (66.3% of mutation rate) following its coinjection with *cas9* mRNA into wild-
22 type embryos (fig. S1). We then constructed a pGGDestISceIEG-1sgRNA vector containing this
23 sgRNA. By injecting this plasmid and I-Sce I enzyme into the blastodisc of 1-cell stage embryos,
24 we created mosaic transgenic sgRNA lines in the Tg(*zpc:zcas9*) or wild-type background. In a
25 representative experiment, we obtained 3 female adults with germ-line GFP expression in a total
26 of 25 F0 fish under the Tg(*zpc:zcas9*) background (12% germ-line transmission rate). After
27 outcross, we observed an average of 16.2% GFP-positive embryos displaying characteristic
28 phenotypes of *Mnanog* mutants (Fig. 2, A-K), with severely delayed epiboly already prominent at
29 the shield stage (Fig. 2, A-D, F-I), and absence of visible dorsoventral and anteroposterior axes at
30 24 hpf (Fig. 2, E and J), reminiscent of previously reported developmental defects (37-39). In
31 contrast, embryos with only sgRNA expression did not show any developmental defects (Fig. 2K
32 and fig. S2).

33 We then randomly selected 10 GFP-positive cleavage stage embryos from two mutant-
34 producing female fish and individually extracted total RNAs to analyze their genotypes. After
35 RT-PCR, the products were cloned into the pZeroback vector, and individual clones were
36 subjected to Sanger sequencing. We found that 2 embryos among the 10 GFP-positive offspring
37 carried mutations in *nanog* transcripts (Fig. 2L). For embryo #1, all 33 sequenced clones had an
38 indel of 8 base pair (bp) insertion and 7 bp deletion, while for embryo #2, all 29 sequenced clones
39 showed an 8 bp deletion (Fig. 2L). Theoretically, as four copies of the haploid genome emerged
40 in the primary oocyte and were edited independently, four different mutant *nanog* RNAs should
41 exist in each maternal mutant embryo. The bias in the emergence of the mutant transcripts may
42 represent a differential RNA stability between these mutant variants. Nevertheless, both types of
43 indels lead to frameshift and premature stop that should produce nonfunctional proteins (Fig. 2L).
44 These results illustrate the feasibility of this transgenic method to generate maternal mutants by
45 only one generation, with less than three months.

46

47 **Multiple sgRNAs increase the efficiency to produce *nanog* maternal mutants**

48 We next designed two additional sgRNAs in the first and second exons of the *nanog* gene.
49 Following verification of their efficiency (fig. S1), the three sgRNAs were cloned tandemly into
50 the pGGDestISceIEG-3sgRNA vector and integrated into the genome of Tg(*zpc:zcas9*) fish. We
51 found that 40% of F0 females (n=10) produced GFP-positive offspring. On average, 38.8% of

52 these GFP expressing Tg(*zpc:zcas9*) embryos displayed typical *Mnanog* phenotypes, while
53 transgenic embryos expressing the three sgRNAs and GFP but without Cas9 did not show any
54 developmental defect (Fig. 2K, fig. S2). The efficiency was hence about 2.5 folds higher than that
55 obtained by just one sgRNA.

56 To further test if the embryos with aberrant phenotypes are bona fide *nanog* maternal mutants,
57 we performed whole-mount immunofluorescence staining on wild-type and GFP-positive
58 embryos at 3 hpf with a commercially available Nanog antibody. As zygotic transcription is not
59 activated at this stage, only maternal Nanog protein exists in these embryos. All wild-type
60 embryos showed a strong Nanog protein signal, with an apparent enrichment in the nuclei (Fig. 2,
61 M-P). However, 46.5% (n=43) of GFP-positive embryos were negative for Nanog antibody
62 staining, indicating loss of maternally deposited Nanog protein in these mutants (Fig. 2, Q-T). To
63 determine the expression of Nanog target genes, we examined *sox17* and *mxtx2* transcripts by
64 whole-mount in situ hybridization (ISH). Sox17 is a transcription factor specifically expressed in
65 the endoderm and the dorsal forerunner cells. Compared to wild-type embryos, *sox17* expression
66 in the endoderm of defective embryos was significantly reduced, and its expression in the dorsal
67 forerunner cells expanded laterally (Fig. 2, U and V). Mxtx2 is another transcription factor
68 expressed exclusively in the yolk syncytial layer (YSL) and is essential for epiboly movement
69 (41). Its expression completely disappeared in presumed *Mnanog* mutant embryos at the sphere
70 stage (Fig. 2, W and X). These disrupted gene expression patterns were identical to those
71 observed in the genuine *Mnanog* mutants (37-39), suggesting that these defects were caused by
72 loss of maternal Nanog function. To further demonstrate the specific loss of maternal *nanog* gene
73 products, we tried to rescue the defective epiboly phenotype by supplementing wild-type *nanog*-
74 *myc* mRNA to GFP-positive embryos (Fig. 2Y). The result showed that there was almost a
75 complete rescue of epiboly defects (Fig. 2Z). Thus, these data demonstrate a significant increase
76 in the efficiency of producing maternal mutants by targeting a single gene with multiple
77 transgenic sgRNAs.

78

79 **Expression of multiple sgRNAs in the oocyte tends to generate large deletions**

80 We next tried to genotype *nanog* maternal mutants created by oocyte expression of multiple
81 sgRNAs. As each oocyte is surrounded by a large number of follicle cells and contains a minimal
82 amount of genomic DNA, it is almost impossible to perform DNA extraction from a single oocyte

83 without contamination by genomic DNA from somatic follicle cells. Hence, we designed a
84 pipeline based on the regulative development of zebrafish early embryos and used RT-PCR
85 analysis to genotype the maternal mutants. GFP-positive embryos were raised to 3 hpf when
86 dozens of cells were aspirated from the blastodisc and subjected to RNA extraction. These cells
87 only express maternal mRNAs because they were collected before zygotic genome activation
88 occurring at 3.5 hpf. Thus, we can genotype the donor embryo by performing RT-PCR analysis of
89 the transcripts of interest. All donor embryos were allowed to develop to appropriate stages for
90 phenotype analysis. If an embryo presents the maternal mutant phenotype, total RNAs from
91 collected cells were extracted, and RT-PCR was performed to amplify the coding sequence (CDS)
92 of concerned transcripts. The resulting PCR products were directly analyzed by agarose gel
93 electrophoresis and further amplified by a pair of nested primers for constructing a mini
94 expression library. The reverse primer sequence is located immediately before the stop codon and
95 ensures in-frame cloning of the open reading frame (ORF) from wild-type mRNA with the six
96 myc epitopes in the pCS2-MT vector. So if mutant sequences with frameshifts or large deletions
97 are cloned, they will not produce any protein with correct molecular weight (see also Materials
98 and Methods and table S1). This library can be examined by sequencing individual clones or can
99 be injected into wild-type embryos to verify if the mutant transcripts produce protein products by
00 western blot analysis (Fig. 3A).

01 Using this strategy, we examined 16 embryos with apparent *Mnanog* phenotypes from 4
02 independent F0 founders. By PCR amplification of *nanog* CDS from collected cells, we found
03 that all these embryos had truncated *nanog* maternal transcripts (Fig. 3B). Some embryos
04 displayed multiple patterns of deletions, as revealed by the presence of PCR products with
05 different sizes. It should be noted that, although maternal *nanog* mRNAs are independently
06 produced by four genomic copies of *nanog* gene during the prophase of meiosis I, not all four
07 mutant maternal transcripts in each embryo could be detected by PCR amplification of cDNA
08 pools. This may be due to the reduced stability of some mutant mRNAs and/or the absence of
09 binding sites for primers to amplify the ORF following unintended deletions. The deletions in
10 *nanog* maternal transcripts were further confirmed by directly sequencing their corresponding
11 PCR products (Fig. 3C). Interestingly, except for deletions spanning the sgRNA targeting sites,
12 we also frequently detected truncated transcripts with large deletions that extended several
13 hundred bp downstream of the sgRNA3 site (Fig. 3C; bands 4-2, 5, 7, 8-1, 9-2, 12-2, 14, 15 and
14 16). This deletion-prone phenomenon was finally confirmed by directly examining genomic DNA
15 in other *Mnanog* mutant embryos, where both sgRNA site-spanning and unusual large deletions

16 were detected on the genome (Fig. 3, D and E). In an extreme situation, we observed a deletion of
17 3.8 kb extending from the 3' region of exon 1 to the beginning of exon 4, whereas there is only
18 1.49 kb between the two extreme sgRNA targeting sites (Fig. 3E; embryo #11). We next
19 examined Nanog protein expression in 3 hpf embryos by western blot analysis. A clear band with
20 molecular weight lower than 60 kDa was detected in a wild-type sample, while it was absent in
21 samples from verified *Mnanog* embryos (Fig. 3F). Consistently, western blot analysis following
22 injection of myc-tagged expression libraries constructed from two of these *Mnanog* mutant
23 embryos into wild-type embryos failed to detect the presence of any protein product (Fig. 3G).

24 This deletion bias of genome editing with multiple Cas9 RNPs in the oocyte was further
25 confirmed by sequencing individual clones (n=57) from the two mutant libraries. We detected
26 four different large deletions in a large proportion of clones, as well as other indels, but not wild-
27 type *nanog* transcripts (fig. S3). The deletion of a large portion of *nanog* transcripts could also be
28 confirmed by ISH using a probe encompassing the two extreme sgRNA sites. We found that one-
29 third of GFP-positive embryos (n=45) were devoid of hybridization signal in a representative
30 experiment, whereas all wild-type embryos were strongly positive (Fig. 3, H-J). After genotyping
31 using collected cells at 3 hpf, ISH analysis of all verified *Mnanog* mutant donor embryos (n=11)
32 clearly showed an absence of staining (Fig. 3K). Thus, ISH experiments could help identify
33 maternal mutant embryos or even mutant oocytes induced by multiple sgRNAs.

34 Because possible off-target sequences of the three sgRNAs are not present elsewhere in the
35 genomic region flanked by F2 and R2 PCR primers (Fig. 3C), it is unlikely that these unintended
36 large deletions identified in *Mnanog* mutant embryos are produced due to off-target effects. This
37 was confirmed by injecting the three Cas9 RNPs into fertilized wild-type eggs. At 24 hpf, we
38 sequenced different genomic regions covered by the large deletion (fig. S4A, numbers 1-11).
39 Genome editing activity was only detected in the vicinity of sgRNA sites, but not at those loci
40 distantly located from different sgRNA target sites (fig. S4B). Hence, these data revealed an
41 apparent feature of the genome editing in zebrafish primary oocytes characterized by the frequent
42 occurrence of on-target site-spanning and unintended large deletions.

43

44 **Maternal Cas9 RNPs fail to edit zygotic wild-type *nanog* allele**

45 As maternally inherited Cas9 RNPs are still possible to mutate the paternal wild-type allele,
46 new mutations may continue to be generated, which will complicate the analysis of maternal
47 phenotypes. We thus tried to test the editing activity of maternal Cas9 RNPs on the wild-type
48 allele from the sperm. To discriminate paternally originated DNA from the maternally inherited
49 allele, we set to analyze *Mnanog* embryos with maternally inherited deletions that prevent the
50 amplification of regions flanking each sgRNA target due to the absence of primer binding sites,
51 so that only the paternal genome can be examined. To identify deletion-bearing *Mnanog* embryos,
52 we crossed mutation-carrying F0 female with wild-type male and extracted genomic DNAs from
53 individual *Mnanog* embryos at 10 hpf, a critical time just before massive embryonic death. By
54 PCR amplification using primers F5 and R3 that should not allow the detection of PCR products
55 in wild-type embryos or mutants carrying small indels due to their large distance on the genome
56 (fig. S5A), we found that 88.9% of *Mnanog* embryos (n=9) had on-target large deletions between
57 the two extreme sgRNA sites (fig. S5B).

58 The site-spanning deletion may result from genome editing activity after fertilization, and this
59 may mislead our judgement for the presence of maternally-inherited deletion alleles. To exclude
60 this possibility, we analyzed the sequencing chromatogram data of these PCR products, and found
61 that they all showed a stringent single-peaked pattern at the joining points after site-spanning
62 deletions (fig. S5, C and D), indicating that the deletion was homogeneous in different embryos.
63 This is not supportive to the raised possibility, because genome editing in different embryonic
64 cells should lead to a heterogeneous deletion pattern. In addition, qPCR analysis using primers
65 flanking different sgRNA sites indicated that they were likely present as haploid alleles in the
66 eight *Mnanog* embryos (fig. S5E), suggesting that only maternal *nanog* allele should have the
67 large deletion.

68 We next analyzed the sgRNA targeting regions from the paternal allele. Sequence analysis of
69 genomic regions around the three sgRNA sites showed that they were indeed wild-type, without
70 any hint of mutation, as shown by the chromatogram data (fig. S5F). Because the wild-type allele
71 could not be descended from the mother as those embryos all displayed maternal mutant
72 phenotype, it must originate from the wild-type sperm. Thus, these data suggest that the
73 expression level of maternally deposited Cas9 RNPs is not sufficient to function on paternal wild-
74 type *nanog* alleles in the early embryo. (The first paragraph of Results say “As *Tg(zpc:zcas9)*
75 zebrafish has adequate maternal Cas9 protein for genome editing in the early embryos”)

76 We then supplemented exogenous Cas9 protein, sgRNAs, or both to *Mnanog* embryos with
77 maternally inherited large deletions, and compared the mutation rates occurring on the paternal
78 *nanog* allele at 10 hpf. Interestingly, moderate mutation rates ranging from 14%-29% were
79 observed at all three sgRNA targets following injection of Cas9 protein alone or synthetic
80 sgRNAs alone (fig. S6). These mutation rates were not saturated because they were significantly
81 lower than the editing efficiency observed after coinjecting Cas9 protein and synthetic sgRNAs at
82 comparable doses (fig. S6). These observations suggest that maternal *nanog* allele should have
83 been mutated in the primary oocytes within a confined period when Cas9 protein and sgRNA
84 expression is high enough to reach a threshold.

85

86 **Generation of *nanog* maternal-zygotic (MZ) mutants**

87 As defective embryos obtained by outcrossing mosaic F0 transgenic fish with wild-type males
88 were indeed maternal mutants of *nanog*, we next tried to obtain maternal-zygotic mutant embryos
89 (MZ*nanog*) through two approaches. First, we crossed a mutation-producing female F0 fish with
90 a *nanog* zygotic homozygous mutant male identified during the study to generate MZ*nanog* in
91 their offspring (fig. S7A). As expected, the resulting genuine MZ*nanog* embryos showed epiboly
92 retardation, wide-spread apoptosis and disrupted pattern formation (fig. S7A). Alternatively,
93 MZ*nanog* mutants could also be obtained by injecting the same set of three Cas9 RNPs into GFP-
94 positive embryos derived from outcross between mosaic F0 transgenic fish and wild-type male,
95 which eliminates the remaining wild-type allele. This strategy is particularly time-saving when
96 adult heterozygous or homozygous mutant male fish are not available. We first expressed three
97 *nanog* Cas9 RNPs in wild-type embryos as a control and did not observe any toxic effect as
98 shown by the normal development of injected embryos (fig. S7, B and C). After injection of these
99 Cas9 RNPs in *Mnanog* embryos, we examined *nanog* transcripts at 10 hpf by sequencing
00 individual clones prepared from RT-PCR products. As expected, we detected 5 wild-type
01 sequences among 34 clones in uninjected *Mnanog* embryos (fig. S7D). However, in injected
02 *Mnanog* embryos, all clones contained frameshift mutations or large deletions, suggesting a near-
03 complete removal of wild-type *nanog* allele in these injected embryos (fig. S7E). This was further
04 supported by western blot analysis because we failed to observe any zygotic wild-type Nanog
05 protein at the shield stage after injecting three Cas9 RNPs into *Mnanog* embryos (fig. S7F).
06 Conversely, we did observe wild-type zygotic Nanog protein in uninjected *Mnanog* embryos at

07 the shield stage (fig. S7F). Phenotypic analyses indicated that MZ*Znanog* mutants generated by
08 these two approaches exhibited similar developmental defects as *Mnanog* embryos (fig. S8).

09

10 **Rapid generation of *ctnnb2* maternal mutants with large deletions**

11 To further test the performance of this oocyte-specific conditional knockout strategy, we
12 designed three highly efficient sgRNAs to simultaneously target *ctnnb2* (β -*catenin2*) gene in
13 exons 3, 4, and 9 (Fig. 4S and fig. S9). Because *ctnnb2* is required for activation of maternal Wnt
14 signaling and is essential for the formation of the dorsal organizer, the disruption of its maternal
15 expression leads to a ventralized phenotype (42, 43). After plasmid construction and transgenesis
16 as described above, two female fish were found to produce GFP-positive offspring (20% germ-
17 line transmission rate, n=10). Examination of their phenotypes indicated that an average of 25.6%
18 GFP-positive embryos displayed various degrees of ventralization at 24 hpf (Fig. 4, A-K). There
19 were no such axis defective phenotypes in embryos only expressing *ctnnb2* sgRNAs and GFP
20 (Fig. 4K and fig. S2). When these ventralized embryos were grouped into four categories (V1-V4)
21 as described (44, 45), we found that 59.8% of them displayed the most severely affected
22 phenotype (V4) with complete absence of dorsal axis (Fig. 4K). This proportion is much higher
23 than the 34% of V4 phenotype reported for *ichabod* mutants that almost completely lack *ctnnb2*
24 transcription (42).

25 We further verified the ventralized phenotype by analyzing the expression of *dharma* and
26 *gooseoid*, two organizer genes directly controlled by maternal Wnt signaling. The result showed
27 a complete loss of *dharma* and *gooseoid* expression in 27.7% and 27.2% of GFP-positive
28 embryos at the dome stage, respectively (Fig. 4, L-O). The ventralized phenotype can also be
29 efficiently rescued by injection of wild-type *ctnnb2-myc* mRNA (200 pg/embryo) into GFP-
30 positive embryos at 1-cell stage (Fig. 4P). All rescued embryos displayed wild-type phenotype,
31 but were not dorsalized. This is probably because overexpression of wild-type β -catenin at such a
32 dose was not sufficient to cause excess activation of maternal Wnt signaling. Indeed, extensive
33 dorsalization only occurred after injecting a constitutively active form of *Ctnnb1* (fig. S10).
34 Altogether, these results demonstrate that *ctnnb2* maternal mutant embryos can be efficiently
35 created at an acceptable efficiency through transgenic oocyte expression of CRISPR/Cas9 and
36 multiple sgRNAs.

37 We next performed RT-PCR analysis of *ctnnb2* coding region in 16 maternal mutant embryos
38 with different degrees of ventralization by using isolated cells. Unintended large deletions were
39 detected in all *Mctnnb2* mutants (Fig. 4, Q and R). Up to this point, 100% F0 founders carrying
40 mutations either in *nanog* or *ctnnb2* gene gave rise to embryos with large genomic deletions. In
41 half of these *Mctnnb2* embryos, there was even a near complete removal of *ctnnb2* coding region,
42 as revealed in PCR products 1-2, 2-2, 4-2, 6, 8-2, 11-2, 14-2 and 15-2 (Fig. 4R). This extremely
43 high frequency of inducing deletion alleles was also directly confirmed by analyzing genomic
44 sequences in ventralized mutant embryos (Fig. 4, S and T). In addition to sgRNA site-spanning
45 deletions, we also detected deletions of huge DNA fragment extending distantly from the sgRNA
46 targeting sites. Notably, the deleted regions covered more than 20 kb, whereas there was only 4.2
47 kb between the two extreme sgRNA sites (Fig. 4T; embryos #14 and #15). Similar to the specific
48 editing of *nanog* locus, large deletions resulted from oocyte-specific conditional knockout of
49 *ctnnb2* were not a consequence of off-target effects, because injection of *ctnnb2* Cas9 RNPs into
50 fertilized wild-type eggs did not lead to editing of genomic regions distantly located from the
51 sgRNA target sites (fig. S11).

52 We then constructed *ctnnb2* mini expression libraries from 4 ventralized embryos with V1-V4
53 phenotypes by using a small group of cells. Sequencing of individual clones did not detect the
54 presence of wild-type transcripts (fig. S12). Consistently, injecting these libraries into wild-type
55 embryos produced no protein product (Fig. 4U), suggesting a complete loss of maternal *ctnnb2*
56 products in either mildly or severely ventralized embryos. Like *Mnanog* mutants generated by
57 multiple sgRNAs, *Mctnnb2* mutants could also be identified among GFP-positive embryos at the
58 sphere stage by ISH using a probe hybridizing the *ctnnb2* coding region between the two extreme
59 sgRNA sites (Fig. 4, V-Y).

60 **Efficient inheritance of large deletions generated by oocyte-specific genome editing**

61 To further demonstrate the high efficiency in generating heritable large-deletion alleles by
62 oocyte transgenic expression of CRISPR/Cas9 and multiple sgRNAs, we compared it with the
63 conventional method by injecting Cas9 protein and sgRNA mixture targeting *nanog* into fertilized
64 wild-type eggs. Injected embryos were raised to adulthood, which were outcrossed for further
65 analysis. Although all founders (n=17) produced mutant offspring, only 17.6% of them gave rise
66 to descendants with large deletions (fig. S13A). We also tested the *ctnnb2* locus and found that
67 only 18.8% of mutant carriers (n=16) produced embryos with large deletion alleles (fig. S13A).
68 Therefore, the germ-line transmission rate of large deletions at *nanog* and *ctnnb2* loci by the

69 conventional method is far lower than the 100% transmission ratio (fig. S13A) observed in
70 oocyte-specific expression of multiple Cas9 RNPs.

71 Moreover, founders generated by the conventional method only produced a tiny proportion
72 (9.7%-12.4%) of offspring bearing large deletions among mutant embryos, much less than the
73 percentage (92.3%-94.1%) following this oocyte-specific conditional knockout (fig. S13B).
74 Sequence analysis indicated that all deletions induced by injection of Cas9 RNPs were sgRNA
75 site-spanning, and we never detected unusual larger truncations as observed in the oocyte-specific
76 genome editing via multiple Cas9 RNPs (fig. S13, C-F). These apparent differences clearly
77 illustrate the high efficiency in generating genomic deletions in developing oocytes using
78 transgenic expression of CRISPR/Cas9 and multiple sgRNAs, at least for the two loci tested here.

79 We next examined the transmission of large deletions in the following generations. The lethal
80 phenotype of *Mnanog* mutants was first rescued by injecting wild-type *nanog-myc* mRNA (150
81 pg/embryo) into GFP-positive offspring (Fig. 5, A and B). Injected embryos were genotyped by
82 RT-PCR analysis performed on a group of cells isolated from the blastodisc at 3 hpf, using
83 primers that specifically bind to the 5' and 3' untranslated regions (UTR) of endogenous *nanog*
84 transcripts, but do not recognize synthetic *nanog* mRNAs (Fig. 5A and table S1). In a
85 representative experiment, we found that 46.7% (14/30) of GFP-positive embryos were rescued
86 *Mnanog* mutants that displayed different patterns of deletions in *nanog* transcripts (Fig. 5, C and
87 D). They were raised to sexual maturation and genotyped by PCR amplification of tail fin
88 genomic DNA using different primer pairs to detect wild-type and small indel alleles (F5 and R7)
89 or large deletions (other primer combinations), respectively (Fig. 5E). The results showed that
90 92.9% of *Mnanog* fish (13/14) had large deletions on the *nanog* locus (Fig. 5F). These deletions
91 were transmitted to the offspring when crossed with wild-type fish (Fig. 5G). In particular,
92 unintended large deletions that extended distantly from the extreme sgRNA site were detected in
93 21.4% (3/14) of rescued *Mnanog* adults (#2, #11 and #14) and were faithfully recovered in
94 descendant embryos (Fig. 5H). These data demonstrate that the genomic deletions generated by
95 oocyte-specific genome editing can be inherited efficiently. Thus, this important deletion-biased
96 trend can be potentially applied to many research purposes such as maternal gene knockouts and
97 functional studies of noncoding regions on the genome.

98

99 **Discussion**

00 We have developed a rapid and feasible approach to inactivating maternal gene functions in
01 the developing oocytes by transgenic expression of multiple Cas9 RNPs. As a proof-of-principle,
02 we successfully created *Mnanog* and *Mctnnb2* mutants with high efficiency. Moreover, we
03 observed an apparent bias to generate large deletion alleles in the primary oocytes by this
04 approach. Thus, these data highlight the effectiveness and rapidness of oocyte-specific multiple
05 Cas9 RNPs for the functional study of maternal factors and illustrate the potential of this method
06 for generating deletions in the genome.

07 Two aspects critically hamper the generation of maternal mutants in zebrafish by the
08 traditional approach. Firstly, obtaining maternal mutants is time-consuming, which generally
09 requires crossing and screening for three generations. Secondly, the occurrence of zygotic lethal
10 or infertile phenotype represents an obstacle in obtaining the next generation. Much effort has
11 been made to circumvent zygotic mutant lethality, such as germ-line replacement, oocyte
12 microinjection in situ, mosaic and BACK approaches (7, 9-14). To some extent, these methods
13 have contributed to the understanding of maternal gene functions. However, they are either
14 technically demanding, time-consuming, or less efficient.

15 The method presented here has several advantages. First, it is much simple and particularly
16 labor-saving. To target a gene of interest, one only has to construct an sgRNA expression vector
17 via a well-established Golden Gate method followed by standard microinjection. In particular,
18 only one generation of fish rearing is required to generate maternal mutant embryos, reducing the
19 time by at least half compared with conventional methods and germ-line replacement. Second, the
20 efficiency in obtaining maternal mutants is satisfactory. By introducing three sgRNAs, we could
21 expect an average maternal mutation rate of more than 25% in GFP-positive F1 offspring. For
22 some F0 females, the percentage of maternal mutant embryos can even exceed 50%. Third, F0
23 female fish can be used repeatedly in their lifespan, although they may produce maternal mutants
24 with a fluctuation of 10% between different spawnings. Fourth, the present method ensures a
25 more efficient removal of maternal products from targeted genes. This efficient removal probably
26 results from the precocious expression of *cas9* driven by the *zpc* promoter, which starts in stage I
27 oocytes. Thus, targeted genes could be inactivated early enough to prevent an eventual production
28 of trace amounts of corresponding proteins. In support of this possibility, we found that *Mctnnb2*
29 mutants generated by this new method presented a more severely ventralized phenotype than the

30 *ichabod* mutants, which may not be utterly devoid of *ctnnb2* maternal products (43). Lastly, but
31 not the least, maternal functions of certain genes, such as *piwill*, *vasa* and *dazl*, can only be
32 studied by the present oocyte-specific conditional knockout strategy, because zygotic mutation of
33 these genes leads to germ cell death before the diplotene stage (46-48). As a result, even using
34 germ-line replacement, transplanted germ cells carrying homozygous mutation for these genes are
35 still unable to get rid of premature death. In this situation, the oocyte-specific conditional
36 knockout strategy may be the only possible way to circumvent this difficulty. Therefore, it will be
37 interesting to obtain maternal mutants of these well-known germ plasm components by the
38 method presented here in a future study.

39 We found a significant trend in generating large deletions by oocyte expression of multiple
40 Cas9 RNPs. For *nanog* and *ctnnb2* genes examined here, 100% of F0 fish with germ-line
41 mutations presented large deletions (over 1 kb in the genome). By comparison, large deletions
42 generated by conventional methods, for example injection of Cas9 RNPs to fertilized eggs or
43 early embryos, are significantly less efficient, with germ-line transmission rates varied from 7.1%
44 to 31.3%, according to published data (49-53). Consistently, only 17.6% to 18.8% of deletion
45 transmission rates were observed by the routine method using the same Cas9 RNPs targeting
46 *nanog* and *ctnnb2* in this study. Moreover, deletion-harboring embryos among mutant siblings are
47 scarce by Cas9 RNPs injection into wild-type embryos. Data from this work and a recent
48 publication (54) showed that only 3.2% to 12.4% F1 offspring contained large deletions, whereas
49 more than 90% ratio was observed in *Mnanog* embryos after the oocyte-specific conditional
50 knockout method presented in this study. We also rescued the lethal phenotype of *Mnanog*
51 embryos, and successfully recovered the large deletions in F1 adults and F2 embryos following
52 the oocyte-specific genome editing strategy. Hence, these results highlight the strong potential of
53 multiple Cas9 RNPs for genome editing in primary oocytes to efficiently generate heritable
54 deletion alleles. This novel transgenic method should have a number of important applications. It
55 could create deletions in the promoters, which should prevent potential genetic compensations
56 that hamper the study of gene functions (55, 56). It may also be helpful to remove transcriptional
57 or post-transcriptional elements from the genome. Therefore, it will be interesting to further
58 evaluate the performance of oocyte-specific multiple Cas9 RNPs in generating even larger
59 deletions covering one or multiple gene loci. An improvement of this system should significantly
60 enhance the tool-kit of functional genomics.

61 The mechanisms underlying this deletion-prone tendency are not clear and need further
62 investigation. The prolonged action of Cas9, the disparate chromatin status, and a distinct DNA

63 repair mechanism for double-strand breaks (DSBs) in early-stage oocytes might collectively
64 contribute to the occurrence of site-spanning and those unusual large deletions. In particular,
65 homologous recombination (HR) DNA repair was reported to dominate in meiosis I (57), while
66 non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ) are
67 more active in primordial germ cells (PGCs) and somatic cells. As a result, DSBs generated in the
68 oocytes might need significantly longer time to be repaired by NHEJ or MMEJ, which may help
69 increase the occurrence of sgRNA site-spanning deletions. However, the unintended large
70 deletions extending well beyond the sgRNA targeting sites observed in this study probably rely
71 on a vigorous exonuclease activity, which does not present in NHEJ and MMEJ mechanisms but
72 does play critical roles in the resection process of HR (58). It is thus conceivable that multiple
73 Cas9 RNPs might disrupt the normal HR process. They might introduce additional DSBs on the
74 repair template or newly synthesized DNA strands during strand invasion and extension, leading
75 to an abortive HR. As a result, the single DNA strand generated during the resection process of
76 HR may be trimmed, and DNA repair might switch to other mechanisms such as MMEJ to
77 generate unintended and complicated patterns of deletion alleles (59). These unintended genomic
78 alterations, termed on-target effects or OnTEs, were demonstrated to happen frequently in
79 embryonic stem cells, induced pluripotent stem cells and early embryos of mouse and human
80 origins (59-65). In zebrafish, however, unintended on-target effects were only extensively
81 observed when performing oocyte-specific genome editing, but seldom happened in early
82 embryos injected with Cas9 RNPs. Thus, it is very likely that DNA repair mechanisms employed
83 to heal CRISPR/Cas9 induced DSBs by zebrafish oocytes may be similar to that in mammalian
84 early embryos, but are somewhat different from somatic cells and PGCs in zebrafish embryos.

85 It should be mentioned that in our oocyte-specific conditional knockout method, transgenic
86 *zcas9* expression may vary among female fish. In fact, we found that the expression level of *zcas9*
87 in some Tg(*zpc:zcas9*) fish was too low to generate mutations in the oocytes or offspring.
88 However, the use of homozygous Tg(*zpc:zcas9*) females could ensure the efficiency and the
89 reproducibility of genome editing in oocytes. In general, Tg(*zpc:zcas9*) adult female fish with
90 sufficient genome editing efficiency can be identified relatively easily. For example, in our
91 previous study, we have injected *bmp2b* sgRNA in the embryos and examined the proportion of
92 dorsalized phenotype (34). Another critical quality control step should be set up before
93 phenotyping GFP-positive embryos. The presence of Cas9 activity could be confirmed by testing
94 the efficiency of *bmp2b* sgRNA in GFP-negative siblings. These measures should guarantee the
95 success of maternal knockout experiments. For versatile applications of this method, further

96 efforts should be made to improve the efficiency of oocyte-specific multiple Cas9 RNPs in
97 genome editing. It has been shown that injection of high amounts of Cas9 protein and multiple in
98 vitro synthesized sgRNAs targeting a single gene in the embryos was efficient to disrupt most of
99 the wild-type alleles and produced phenotypes as observed in homozygous zygotic mutants (35,
00 53). Thus, there is a possibility that suboptimal expression of Cas9 protein or sgRNAs in
01 transgenic lines may limit the efficiency of oocyte conditional knockout. Indeed, our data clearly
02 showed that the expression level of maternally inherited Cas9 RNPs was below a threshold for
03 efficient mutagenesis on the wild-type allele from the sperm, demonstrating that the method in its
04 present form is highly specific for knockout of maternal factors without affecting zygotic gene
05 products. In another aspect, however, it also highlights a promising potential to increase the
06 efficiency of this and other conditional knockout systems employing Cas9 RNPs by improving
07 the expression level of Cas9 protein and sgRNAs. It is possible to increase *cas9* transcription via
08 either gal4-UAS or Suntag system (31, 66). At the same time, modification of the *cas9* sequence
09 may help enhance its translation efficiency. Alternatively, sgRNA expression could be improved
10 by strong conventional promoters followed by maturation via Csy4, ribozyme or tRNA
11 processing system (67-69). These procedures could likely increase the efficiency in generating
12 maternal mutants for single or even multiple genes.

13 In summary, our work provides evidence that maternal gene inactivation can be achieved by
14 oocyte transgenic expression of Cas9 and sgRNAs in an important time-saving and deletion-prone
15 manner. The accessibility of this approach should open a new avenue for large-scale functional
16 screening of maternal factors in zebrafish.

17

17

18 **Materials and Methods**

19 **Zebrafish husbandry and ethic statement**

20 Wild-type and Tg(*zpc:zcas9*) fish were raised at 28.5 °C in standard housing systems
21 (Haisheng). Offspring from Tg(*zpc:zcas9*) fish were screened for genome editing efficiency by
22 injecting *bmp2b* sgRNA followed by analyzing the dorsalized phenotype at 11 hpf (34). Only
23 female fish with more than 50% of dorsalized rate in their offspring were kept for breeding and
24 maternal knockout experiments. All experiments were designed and performed following the
25 ARRIVE guidelines issued by the Ethics Committee for Animal Research of Life Science of
26 Shandong University (permit number SYDWLL-2018-05).

27

28 **Design and efficiency of sgRNAs**

29 The sgRNAs for *nanog* and *ctnnb2* were designed via online software CRISPRscan (40) and
30 listed in table S1. Their efficiency was tested before the construction of sgRNA expression
31 vectors. DNA templates for sgRNAs were synthesized by fill-in PCR. After in vitro transcription,
32 the purified sgRNA (150 pg) was injected along with zebrafish-codon-optimized *cas9* mRNA
33 (200 pg) into 1-cell stage embryos. At 10 hpf, injected embryos were lysed in 50 mM NaOH for
34 20 min at 95 °C, followed by neutralization using 1/10 volume of 1 M Tris-HCl (pH 7.5). The
35 DNA fragment containing the sgRNA targeting site was amplified by PCR from the lysate and
36 subjected to Sanger sequencing. Sequencing chromatogram files were uploaded to [http://iai-gec-](http://iai-gec-server.iai.kit.edu/index.php)
37 [server.iai.kit.edu/index.php](http://iai-gec-server.iai.kit.edu/index.php) or <https://ice.synthego.com/#/> (recommended) to quantify the
38 efficiency of the sgRNA.

39

40 **Construction of plasmids for transgenic expression of sgRNAs**

41 pU6x:sgRNA plasmids were constructed as previously described (18). Briefly, primers
42 containing sense and antisense sequences of sgRNA protospacer were synthesized and annealed.
43 The sequence of sense primer is TTC plus G(N)₁₉, where G(N)₁₉ represents the protospacer, while
44 the sequence of antisense primer is AAAC plus reverse-complement sequence of (N)₁₉. Sense and
45 antisense primers were diluted to 100 μM and mixed as follow: 1 μL sense primer, 1 μL antisense
46 primer, 2 μL 10x NEB buffer 2.1 and 16 μL H₂O. The mixture was incubated in a PCR
47 thermocycler with the following program: 95 °C for 15 mins, slow ramping to 50 °C at 0.1 °C/s, 50
48 °C for 10 mins, and cooling quickly to 4 °C. The annealed primers (1 μL) were then mixed in a 10
49 μL system with 100 ng pU6x:sgRNA empty plasmid (pU6a:sgRNA#1, pU6a:sgRNA#2 or

50 pU6b:sgRNA#3), 1 μ L 10x Cutsmart buffer, 1 μ L 10x T4 ligase buffer, 0.3 μ L T4 ligase, 0.3 μ L
51 *BsmB* I, 0.2 μ L *Pst* I and 0.2 μ L *Sal* I. They were incubated in a PCR thermocycler using the
52 following program: 37 $^{\circ}$ C for 20 mins and 16 $^{\circ}$ C for 15 mins for 6 cycles, 37 $^{\circ}$ C for 10 mins, 55 $^{\circ}$ C for
53 15 mins and 80 $^{\circ}$ C for 15 mins. The ligation products were then used to transform DH5 α *E. coli*.
54 After spreading on *Spec*⁺ plates, positive clones were identified by PCR using the M13 forward
55 and the protospacer antisense primers. The corresponding plasmids were extracted for further
56 construction of the final transgenic vector.

57 Golden Gate assembly was used to ligate single or three sgRNA expression cassettes into the
58 pGGDestISceIEG-1sgRNA or pGGDestISceIEG-3sgRNA backbone plasmid. Here we take the
59 cloning of three sgRNAs as an example. The pU6a:sgRNA#1, pU6a:sgRNA#2 and
60 pU6b:sgRNA#3 plasmids (100 ng each) were mixed with 2 μ L 10x Cutsmart buffer, 2 μ L 10x T4
61 ligase buffer, 50 ng empty pGGDestISceIEG-3sgRNA, 1 μ L T4 DNA ligase and 1 μ L *Bsa* I in a
62 20 μ L reaction mixture. It is incubated using the following PCR program: 37 $^{\circ}$ C for 20 mins and
63 16 $^{\circ}$ C for 15 mins for 3 cycles, and then 80 $^{\circ}$ C for 15 mins. The ligation products were transformed
64 to DH5 α *E. Coli* and plated on Amp⁺ LB medium. Positive clones were identified by colony PCR
65 using primers: 5'-TTCTTGTTTAAGCTTTTAATCTCAAAAAC-3' and 5'-
66 GGCTGTTTACATCTGATAGTGG-3'. The annealing primers for *nanog* and *ctnnb2* sgRNAs are
67 listed in table S1. pGGDestISceIEG-1sgRNA plasmid was constructed similarly. The only
68 difference lies in the ligation mixture components, where only 100 ng pU6a:sgRNA#1 was added,
69 and the pGGDestISceIEG-3sgRNA should be replaced with pGGDestISceIEG-1sgRNA.

70 71 **I-Sce I-mediated transgenesis**

72 A mixture (1-2 nL) containing 10 ng/ μ L pGGDestISceIEG-1sgRNA or pGGDestISceIEG-
73 3sgRNA plasmid, 1 U/ μ L I-Sce I (NEB), and 0.5x CutSmart buffer (NEB) was injected into the
74 blastodisc of 1-cell-stage embryos (36). At 4 dpf, the larva showing strong and extensive green
75 fluorescence, reflecting early integration event, were picked up and raised to adulthood. The
76 overall germ-line transmission rate of I-Sce I-mediated transgenesis may depend on whether an
77 early integration event occurs in the embryos. As shown in the present study, the germ-line
78 transmission rates varied from 12% to 40%. In fact, if only embryos with wide-spread GFP
79 expression at 4 dpf are selected, the germ-line transmission rate will be normally higher than
80 50%. Typically, injection of 200 embryos will produce about 10-20 embryos with early
81 integration and will be sufficient to screen for maternal mutant-carrying founders.

82

83 **Quality control before screening of maternal mutants**

84 GFP-negative sibling embryos from transgenic F0 female founders were injected with 100 pg
85 *bmp2b* sgRNA. The phenotype of GFP-positive embryos was further analyzed only when their
86 GFP-negative counterparts were efficiently dorsalized.

87
88 **Rescue of mutant phenotypes by wild-type mRNAs**

89 The *nanog* CDS was amplified and inserted into the *Bam* HI and *Cla* I sites of the pCS2-MT
90 vector using T4 DNA ligase. The *ctnnb2* CDS was inserted into the *Bam* HI site of the pCS2-MT
91 vector by Gibson assembly. We also constructed mouse ΔN -*ctnnb1*-pCS2 for comparison of
92 dorsalizing effects. PCR primers are listed in table S1. Synthetic mRNAs were in vitro transcribed
93 using the mMESSEGE mMACHINE SP6 Kit (Invitrogen, AM1340). For rescue, 200 pg *nanog*-
94 *myc* or *ctnnb2-myc* mRNA was injected into 1-cell-stage GFP-positive embryos. Their
95 phenotypes were analyzed at the bud stage (*Mnanog*) or 24 hpf (*Mctnnb2*)

96
97 **Cell isolation, RNA extraction, and cDNA synthesis**

98 To check the genotype of maternal mRNAs, we aspirated about 30-40 cells from 1k-cell stage
99 embryos using a pneumatic microinjector (12). They were lysed in 200 μ L Trizol, and total RNAs
00 were extracted by adding 40 μ L chloroform and precipitated by isopropanol. To facilitate RNA
01 precipitation, 1 μ L glycogen solution (20 mg/mL) was added to each sample. Then cDNAs were
02 synthesized using the First-Strand cDNA Synthesis kit (Transgene, AT301) and subjected to PCR
03 amplification using primers listed in Supplementary Table 1.

04
05 **Gel analysis and sequencing**

06 The CDS in genes of interest were amplified by PCR from embryos with defective phenotypes
07 and subjected to agarose gel electrophoresis. DNA fragments of different sizes were sequenced
08 and aligned with the wild-type sequence using SnapGene software.

09
10 **Library construction and single clone sequencing**

11 CDS amplicons from intact embryos or isolated blastomeres were ligated into pCS2-MT
12 vector in-frame with the 6 myc epitopes. The reverse primer used for library construction was
13 designed immediately before the stop codon. A 15 bp 5'-homology arms was designed for Gibson
14 ligation and ensure the in-frame connection between the open reading frame (ORF) of the wild-
15 type mRNA and the 6 myc epitope sequence in the pCS2-MT vector. Following transformation in

16 *E. coli*, individual clones were subjected to Sanger sequencing. The primers used for constructing
17 the library were listed in table S1

18

19 **Western blot**

20 The plasmid library (30-40 pg) was injected into 1-cell stage embryos. At 12 hpf, at least 30
21 injected embryos were dechorionated, and the yolk was removed. Cell pellets were then lysed in
22 the lysis buffer (100 mM NaCl, 0.5% NP-40, 5 mM EDTA, 10 mM Tris-HCl pH 7.5) containing
23 a cocktail of protease inhibitors and boiled in Laemmli sample buffer. Protein sample were
24 subjected to polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and
25 probed with anti-Myc (1:1000, Abcam, ab32) or anti- α -Tubulin (1:1000, GeneTex, GTX124303)
26 antibodies. Following a similar procedure for yolk removal and protein extraction, we also used
27 anti-Nanog (1:500, ZDB-ATB-191003-3, GTX132491, GeneTex) to detect endogenous Nanog
28 protein, where anti- β -actin (1:5000, 66009-1-Ig, Proteintech) served as a loading control.

29

30 **Whole-mount immunofluorescence staining**

31 Embryos were fixed at 3 hpf in 4% paraformaldehyde. Antigen retrieval was performed by
32 treating embryos in 0.1M Tris-HCl (pH 9.5) for 15 mins at 70 °C. The embryos were then
33 subjected to immunofluorescence staining with anti-Nanog (1:500) and counterstained with
34 Hoechst 33342 (Cell Signaling Technology, 4082S) as described (70). Images were acquired
35 using a spinning disc confocal microscope (Olympus SpinSR10) with its Z-stack function.

36

37 **RNA probe design and in situ hybridization**

38 ISH was performed as previously described (45). Probes for *dharma* and *gooseoid* were
39 reported previously (45). The coding regions for *sox17* and *mxtx2* and cDNA sequences within
40 the two extreme sgRNA targets for *nanog* and *ctnnb2* were amplified by PCR and cloned in
41 pZeroback vector. Digoxigenin-labeled probes were in vitro transcribed using T7 or SP6 RNA
42 polymerase in the presence of Dig-labelling mix (Roche).

43

44 **Quantitative PCR**

45 Wild-type embryos and *Mnanog* mutants were lysed at 10 hpf, and their genomic DNAs were
46 extracted. Quantitative PCR was performed using 2x M5 HiPer SYBR Premix (MF787-01, Mei5)
47 on a Q1000 Real-Time PCR System (LongGene). Primers for amplifying *nanog* sgRNA target
48 sites and *dmd* genomic regions are listed in table S1. The $2^{-\Delta\Delta Ct}$ method was employed to estimate

49 ploidy at these loci. The average copy number of these amplicons in wild-type embryos was
50 normalized as two.
51

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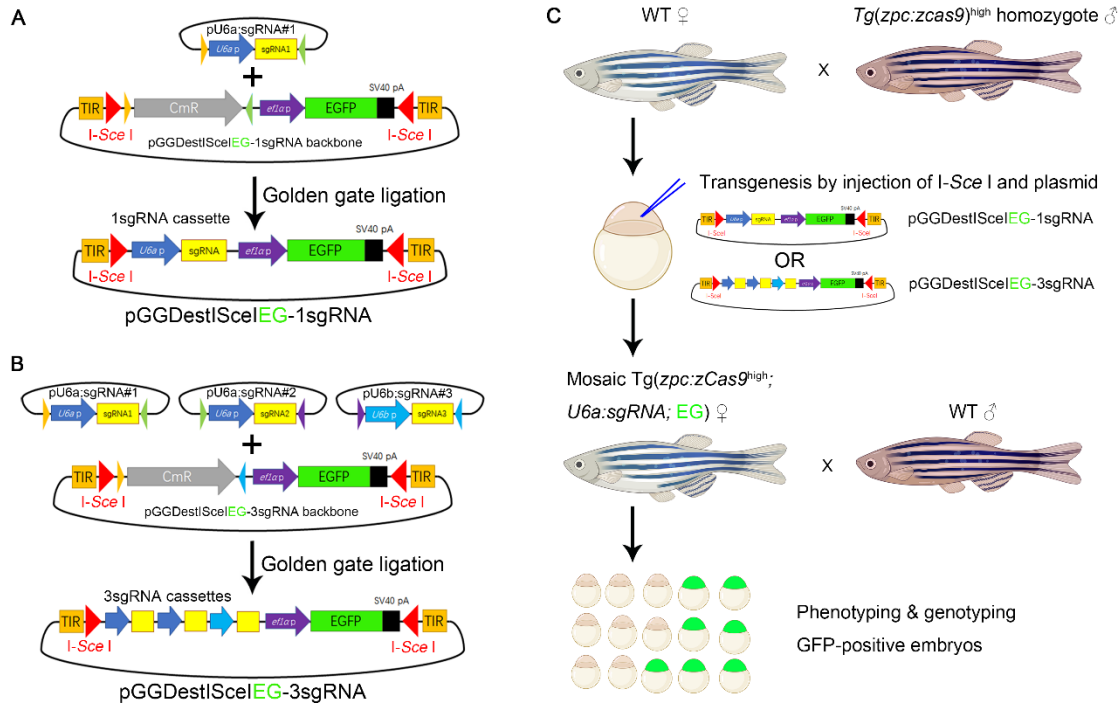
16 **Author contributions:** CZ and MS conceived the idea. CZ, TL, YZ, JL, IT, FW, AC, JW, ZZ
17 and MS performed the experiments. CZ, YZ, DS and MS analyzed and validated the data. MS
18 and DS acquired funding. MS supervised the work and wrote the original draft. MS, DS and CZ
19 reviewed and edited the manuscript.

20 **Competing interests:** There is no competing interest.

21 **Data and materials availability:** All data needed to evaluate the conclusions in the paper are
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23 study are available from the corresponding author upon reasonable request.

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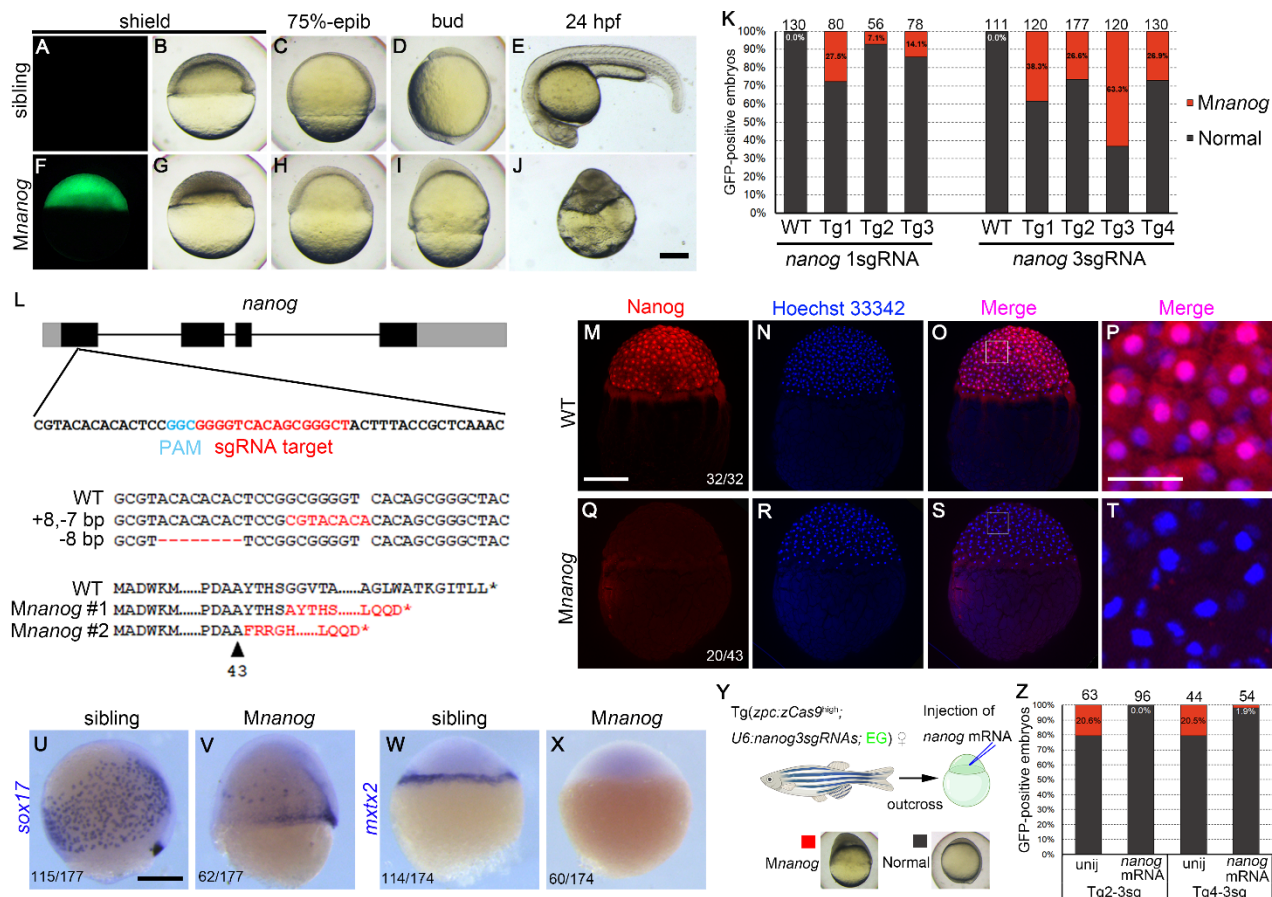
25 **Figures and Tables**

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28 **Figure 1. Experimental design of a CRISPR/Cas9 system to generate maternal mutants in**
 29 **F1 embryos.** (A) Construction of pGGDestISceIEG-1sgRNA plasmid that contains a single
 30 sgRNA expression cassette and a GFP expression module driven by the *efla* promoter. I-Sce I
 31 restriction sites and Tol2 inverse repeat sequences (TIR) are designed to flank sgRNA and GFP
 32 sequences for transgenesis. (B) Construction of the transgenic pGGDestISceIEG-3sgRNA vector
 33 expressing three tandem sgRNAs via Golden Gate ligation. (C) The pipeline to generate maternal
 34 mutants in F1 embryos. The sgRNA expression cassette is introduced into *Tg(zpc:zcas9)* embryos
 35 by I-Sce I-mediated transgenesis, and the phenotype of the resulting GFP-positive F1 embryos is
 36 examined to identify developmental defects. CmR represents the Chloramphenicol resistant gene,
 37 while EG highlighted in green designates the GFP expression cassette driven by the *efla*
 38 promoter.

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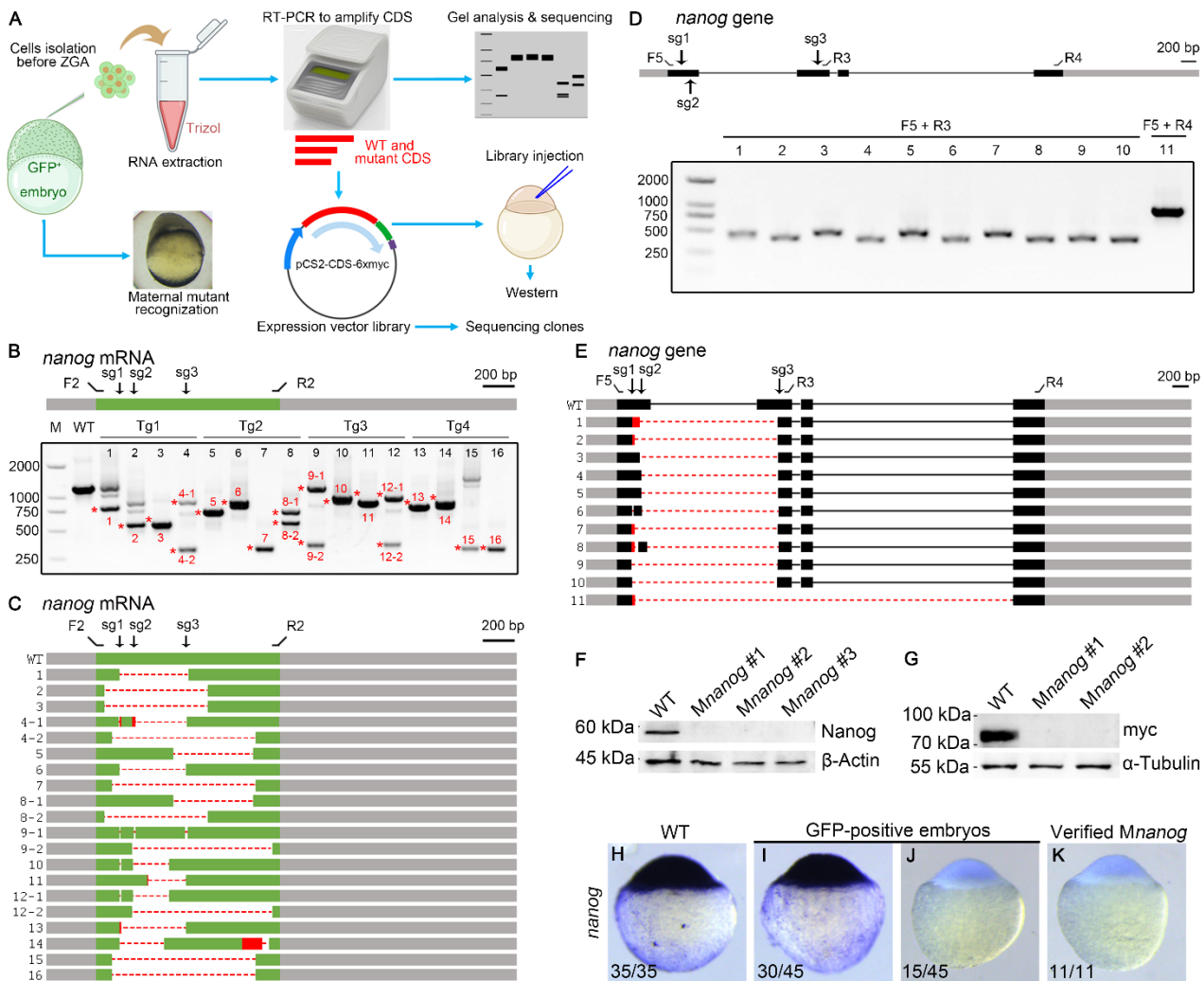


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41 **Figure 2. Rapid generation of *nanog* maternal mutants by transgenic expression of one or**
 42 **three sgRNAs. (A-E)** GFP-negative F1 sibling embryos developed normally. **(F-J)** Typical
 43 *Mnanog* defective phenotypes observed among GFP-positive embryos. All embryos are lateral
 44 view with the dorsal region on the right **(A-D, F-I)** or upward **(E and J)**. **(K)** Stacked columns
 45 show the ratio of *Mnanog*-like defective embryos among GFP-positive offspring in *Tg(zpc:zcas9)*
 46 background. Higher efficiency in generating maternal mutants was observed by expressing three
 47 sgRNAs compared to that expressing one. Numbers on the top of each column represent the total
 48 GFP-positive embryos scored. WT, wild-type fish; Tg1 to Tg4, different mutation-carrying F0
 49 founders. **(L)** Single sgRNA expression in *Tg(zpc:zcas9)* background leads to indel in *nanog*
 50 maternal transcripts, resulting in frame-shift and premature termination that produce
 51 truncated and nonfunctional proteins. The four exons of *nanog* are represented by black boxes,
 52 with grey indicating untranslated regions. Mutant transcripts identified in two independent
 53 embryos at 3 hpf. The dashed line corresponds to deleted nucleotides; red bases indicate
 54 insertions. Red letters in protein sequences indicates amino acid residues translated after
 55 frameshift mutation. Arrowhead represents the position of amino acid in wild-type protein. **(M-P)**
 56 Nuclear accumulation of Nanog protein in 3 hpf wild-type embryos revealed by

57 immunofluorescence and confocal imaging. (Q-T) Absence of Nanog protein expression in 3 hpf
58 *Mnanog* embryos. (P) and (T) are magnified images of framed regions in (O) and (S). Hoechst
59 33342 (blue) was employed to stain nuclei. (U-X) ISH analysis shows the expression of *sox17* at
60 7.5 hpf and *mxtx2* at the sphere stage. Lateral view with dorsal region on the right (U and V). (Y)
61 The procedure to rescue *Mnanog* phenotype by mRNA injection. (Z) Stacked columns show a
62 nearly complete rescue of *Mnanog* mutant defects after injection of the wild-type *nanog-myc*
63 mRNA. Numbers on the top represent total embryos scored from two experiments. Scale bars: 50
64 μm for (P) and (T); 250 μm for others.

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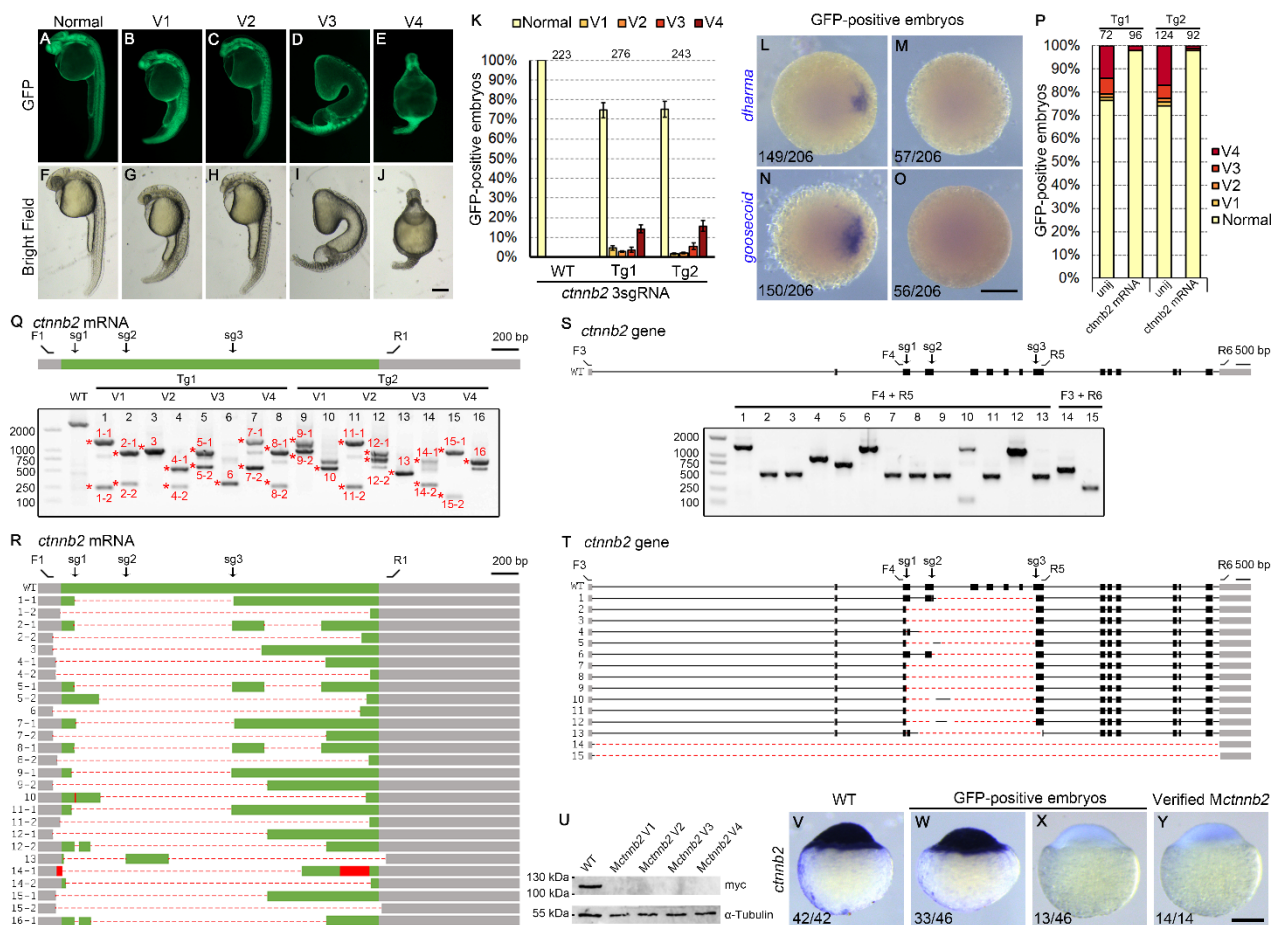
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Figure 3. The deletion-biased tendency in *Mnanog* mutant embryos induced by multiple *Cas9* RNPs. (A) Illustration of the strategy combining phenotyping and genotyping. A small group of cells are isolated from GFP-positive embryos at 3 hpf and stored in Trizol. When donor embryos developed maternal mutant phenotype, total RNAs from corresponding cells are extracted and subjected to RT-PCR analysis. Primers are designed to amplify the entire wild-type CDS. PCR products are either analyzed directly by gel electrophoresis or subcloned into pCS2-MT vector, generating a mini expression library. A control library is made from the CDS region amplified from a wild-type embryo using the same set of primers. These primers are designed to ensure the in-frame ligation of the wild-type CDS and the 6 myc epitopes. The libraries are either injected into the embryos to check protein expression or subjected to single clone sequencing. (B) RT-PCR analysis of *nanog* CDS region from 16 *Mnanog* mutant embryos shows the presence of truncated transcripts. Red asterisks and numbers indicate PCR products subjected to Sanger sequencing. F2 and R2 represent primers used for amplification of the CDS region. The positions

81 of sgRNA targeting sites are marked as sg1, sg2, and sg3. Grey boxes indicate untranslated
82 regions, and the green box represents the CDS region. (C) Diagram summarizes the sequencing
83 results. Dashed lines represent deletions, while red boxes indicate insertions. (D) Analysis of
84 genomic deletions in *Mnanog* mutant embryos using different primer sets as displayed on the
85 *nanog* gene. Grey and black boxes designate UTR and CDS regions of exons, respectively; thin
86 lines indicate introns. (E) Sequencing of PCR products reveals various patterns of genomic
87 deletions. (F) Absence of Nanog protein in verified *Mnanog* embryos at 3 hpf. Total proteins
88 were obtained from four embryos in each condition. (G) Injection of expression libraries
89 constructed from two *Mnanog* mutants into wild-type embryos produced no protein product. (H-
90 K) ISH was used to examine the absence of *nanog* transcripts among GFP-positive embryos and
91 in *Mnanog* embryos at the sphere stage. *Mnanog* mutant embryos were confirmed by genotyping
92 of their isolated cells. Scale bar: 250 μm .

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96 **Figure 4. Generation of *ctnnb2* maternal mutants and large deletions by transgenic**
 97 **expression of multiple Cas9 RNPs. (A-J)** Phenotypes of GFP-positive embryos simultaneously
 98 expressing three sgRNAs targeting *ctnnb2*. **(K)** Statistical analysis of ventralized phenotypes in
 99 GFP-positive offspring derived from two mutation-carrying Tg(*zpc:zcas9*) F0 fish. Numbers
 00 designate total embryos analyzed. Error bars indicate standard deviations (SD) from three
 01 independent spawnings. **(L-O)** Absence of *dharma* and *goosecoid* expression in GFP-positive
 02 *Mctnnb2* embryos. Scale bars: 250 μ m. **(P)** The ventralized phenotype in GFP-positive *Mctnnb2*
 03 embryos can be rescued by overexpression of the wild-type *ctnnb2-myc* mRNA. Numbers on top
 04 of each column represent total embryos scored. Tg1 and Tg2 are two independent mutation-
 05 carrying F0 fish. **(Q)** RT-PCR analysis of the CDS region from 16 *Mctnnb2* mutant embryos.
 06 Wild-type (WT) embryos served as a control. Deletion alleles were present in all mutant embryos
 07 tested. V1 to V4 indicate the degrees of ventralized phenotypes, as shown in **(A-J)**. Asterisks and
 08 numbers designate PCR products subjected to Sanger sequencing. F1 and R1 are primers used to
 09 amplify the CDS region of *ctnnb2* transcripts. The positions of the three sgRNA targeting sites are
 10 indicated as sg1, sg2, and sg3. Grey boxes indicate UTRs; the green box represents the CDS
 11 region; dashed lines represent deletions; red boxes indicate insertions. **(R)** Sequencing results of

12 PCR products show extensive deletion events. (S) Analysis of deletion events in the genome of
13 *Mctnnb2* mutant embryos using indicated primer sets. Grey boxes indicate UTRs, and black
14 boxes represent coding regions. Primer positions and sgRNA targeting sites are indicated on the
15 wild-type allele. (T) Sequencing results show different deletion patterns at the *ctnnb2* locus. (U)
16 No Ctnnb2 protein product was produced after injecting expression libraries from *Mctnnb2*
17 embryos with V1-V4 phenotypes. (V-Y) ISH was used to examine the absence of *nanog* mRNA
18 among GFP-positive embryos and in *Mnanog* embryos at the sphere stage. *Mnanog* mutant
19 embryos were confirmed by genotyping of isolated cells (Y). Scale bars: 250 μ m.

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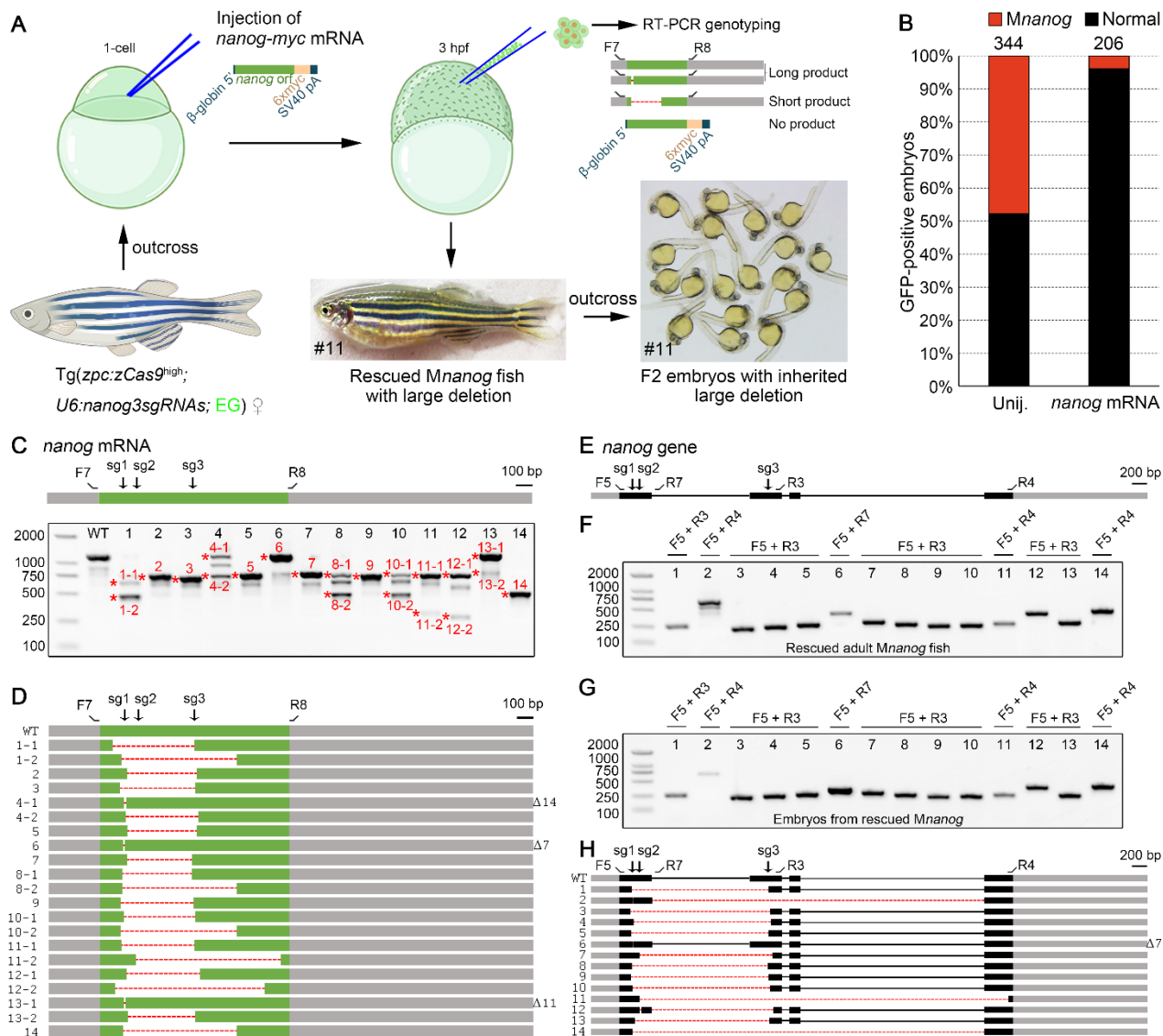


Figure 5. Highly efficient inheritance of large deletions. (A) Diagram demonstrates the rescue of *Mnanog* lethal phenotype by injection of wild-type *nanog-myc* mRNA, identification of rescued maternal mutants, and the resulted deletion-containing F1 fish and F2 embryos. A pair of primers was designed to specifically amplify the CDS of endogenous *nanog* mRNA. The rescued F1 adult female fish shown in the diagram carries an inherited unintended large deletion in one allele. Thus, F2 offspring derived from outcross with a wild-type fish developed normally. (B) Stacked bars show the efficient rescue of *Mnanog* lethal phenotype by injecting 150 pg/embryo *nanog-myc* mRNA. (C) RT-PCR analysis of *nanog* coding region in 14 rescued *Mnanog* embryos. Asterisks and numbers designate bands subjected to Sanger sequencing. In the schema of *nanog* mRNA, grey boxes indicate UTRs; the green box represents the CDS region. F7 and R8 represent the primer pair used for amplification of *nanog* ORF. The three sgRNA target sites are

34 marked by sg1, sg2 and sg3. **(D)** Sequencing results of PCR products as indicated in **(C)**. Dashed
35 lines represent deletions. Numbers of deleted nucleotides in small indels are indicated on the
36 right. **(E)** Schema of *nanog* gene with sgRNA target sites and genotyping primers indicated. Grey
37 boxes indicate UTRs, and black boxes represent coding regions. **(F)** PCR analysis of deletions in
38 14 rescued *Mnanog* mutant adults. Primer pairs are indicated on the top of each lane and their
39 positions can be found in **(E)**. **(G)** Similar patterns of genomic deletions in offspring derived from
40 outcrosses of 14 rescued F1 *Mnanog* mutant adult fish with wild-type fish. **(H)** Sequence analyses
41 of PCR products from 14 rescued F1 *Mnanog* adults. Their outcrossed offspring displayed
42 identical deletions in the genome.