Efficient TALEN-mediated gene targeting of chicken primordial germ cells

Lorna Taylor[1], Daniel F. Carlson[2], Sunil Nandi[1], Adrian Sherman[1], Scott C. Fahrenkrug[2], Michael J. McGrew[1*]

[1]The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh, Easter Bush Campus, Midlothian, EH25 9RG, UK
[2]Recombinetics Inc, 1246 University Avenue West, Suite 300, Saint Paul, MN 55104 USA

[*]to whom correspondence should be addressed.

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Abstract

In this work we use TALE nucleases (TALENs) to target a reporter construct to the *DDX4* (vasa) locus in chicken primordial germ cells. Vasa is a key germ cell determinant in many animal species and is posited to control avian germ cell formation. We show that TALENs mediate homology directed repair of the *DDX4* locus on the Z sex chromosome at high (8.1%) efficiencies. Large genetic deletions of 30kb encompassing the entire DDX4 locus were also created using a single TALEN pair. The targeted PGCs were germ line competent and were used to produce *DDX4* null offspring. In *DDX4* knockout chickens, PGCs are initially formed but are lost during meiosis in the developing ovary leading to adult female sterility. TALEN-mediated gene targeting in avian primordial germ cells is therefore an efficient process.

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Summary statement

We used gene editing vectors to efficiently disrupt the *DDX4* gene in chicken germ cells. The gene targeted germ cells were used to produce chicken lacking *DDX4*. In contrast to mammals we found that hens containing the mutation were sterile and *DDX4* is needed for proper oocyte differentiation.

Introduction

The chicken embryo is an established model for studying the genetic pathways regulating early patterning events and lineage commitment and also provides a comparative model for mammalian embryogenesis (Stern, 2005). In addition, the chicken is a key agricultural commodity accounting for 30% of the worldwide meat production and 1.2 billion eggs yearly (FAOSTAT, 2012). Therefore, the ability to precisely genetically edit the chicken genome will not only allow the investigation of key developmental signalling pathways in avian species but also examine genes involved in egg production, disease susceptibility and resistance to promote the sustainability and biosecurity in both livestock and poultry production (Tizard et al., 2016; Whyte et al., 2016).

Genetic manipulation of avian species has lagged behind mammals due to the complexity of the avian egg and the lack of germ line competent embryonic stem cell lines (Hunter et al., 2005). However, in sharp contrast to other vertebrate species, primordial germ cells (PGCs) from the chicken can be propagated *in vitro* in suspension and maintain germ line competence when transplanted back into donor embryos (van de Lavoir et al., 2006).

Vasa, a dead box RNA helicase originally identified in *Drosophila,* is essential for proper germ cell formation in multiple species (Schupbach and Wieschaus, 1986; Komiya et al., 1994; Gruidl et al., 1996; Kawasaki et al., 1998; Knaut et al., 2000). Moreover, in *Drosophila*, C. *elegans* and *D. rerio*, vasa is essential for oogenesis whereas in mice and basally branching insects, vasa homologues are necessary for male germ cells to progress through spermatogenesis (Kawasaki et al., 1998; Styhler et al., 1998; Tanaka et al., 2000; Kuramochi-Miyagawa et al., 2010; Ewen-Campen et al., 2013; Hartung et al., 2014). The divergent role of vasa between different species is likely due to the differing mechanisms of PGC specification adopted by these species. In *Drosophila, C. elegans* and *D. rerio* germ cell fate is acquired through the inheritance of maternal determinants, vasa being one of these, and PGCs are present at the start of embryogenesis. Contrastingly, in mice, Urodele amphibians and field crickets, PGCs arise from the mesoderm during mid-embryogenesis as a result of signalling cues (reviewed in (Extavour and Akam,

2003)). Chicken vasa homologue (CVH) (DDX4) marks the chicken germ cell lineage at the earliest stages of embryonic development and therefore is hypothesised to be a maternal determinant for formation of the germ cell lineage (Tsunekawa et al., 2000). As a consequence, we would expect vasa to play a role in oogenesis in the chicken.

Here we use TALENs to knockout the *DDX4* (*CVH* (vasa)) locus in chickens with the aim to demonstrate efficient targeting of genes important for development of the germ cell lineage. TALE (transcriptional activator-like effectors) nucleases (TALENs) are synthetic transcription factors that can be modularly assembled into functional dimers that will target and cleave specific DNA sequences (usually 14-17bp sequences for each module) in the target genome (Bogdanove and Voytas, 2011). Genomic cleavage can result in non-homologous end joining (NHEJ) that can cause small deletions or insertions (indels) at the target cleavage site. Introduction of a region of homology surrounding the cleavage site can result in homology directed repair (HDR) that will lead to incorporation of an exogenous DNA sequence into the target site of the genome. Classical gene targeting by homologous recombination has been demonstrated in cultured chicken PGCs (Schusser et al., 2013). By using CRISPR/Cas9, however, the frequency of homologous recombination in PGCs was greatly increased (Dimitrov et al., 2016). Both CRISPR and TALEN vectors have been used to generate indels in chicken PGCs and the resulting offspring (Park et al., 2014; Oishi et al., 2016), and to modify the genome of other vertebrate species (Tesson et al., 2011; Carlson et al., 2012; Tan et al., 2013; Zu et al., 2013; Carlson et al., 2016).

Using TALEN-mediated homologous recombination we efficiently targeted the DDX4 locus. In contrast to the phenotype previously observed in mice, female chickens were sterile and contained no detectable follicles post-hatch. Examination of early embryos revealed that the germ cell lineage was initially formed but female PGCs were subsequently lost during meiosis. This study demonstrates the utility of TALENs in genome targeting of poultry and the conserved function of the *DDX4* gene in germ cell development and oogenesis.

Results

To target the *DDX4* locus we utilised TALEN-stimulated HDR to recombine a GFP-2a-puromycin transgene targeting vector into the *DDX4* locus, replacing exon 2 and 3 of the endogenous *DDX4* locus (Fig. 1A). A TALEN pair was designed that cleaved exon 2 of the *DDX4* locus immediately downstream of the ATG start codon. The targeting vector contained homology arms of 2.9 and 4.3 kb in length and fused a GFP-2a-puromycin reporter gene to the endogenous ATG codon. The correctly targeted locus expresses GFP-2a-puromycin under control of the endogenous *DDX4* regulatory regions and a polyA termination signal terminates transcription prior to exon 4. The TALEN pair and the targeting vector were first transfected into PGCs and transiently selected (48 hours) with puromycin to eliminate untransfected cells. PGCs were subsequently propagated in culture for two weeks and examined by flow cytometry to identify cells stably expressing the GFP transgene (Fig. 1B). PGCs transfected with the targeting vector alone did not express GFP. This is consistent with previous results which demonstrated that randomly integrated DNA vectors lacking insulator elements could not be stably selected in cultured PGCs (Leighton et al., 2008; Macdonald et al., 2012b). In contrast, 8.1% of the PGCs stably expressed GFP when co-transfected with the TALEN pair. To demonstrate that the GFP transgene was correctly integrated into the *DDX4* locus, a Southern blot analysis was performed. Several independent transfections of male and female PGC lines were carried out and for each transfection the PGCs were selected with puromycin and genomic DNA was isolated (Fig. 1C). The *DDX4* gene is located on the Z chromosome and male ($Z^{GFP}Z$) PGCs contain two chromosomal copies of the gene whereas female ($Z^{GFP}W$) PGCs are hemizygous and contain a single copy of the *DDX4* gene. Multiple targeting events are likely to occur during each transfection as clonal colonies were not selected. The intensity of the bands in the Southern blot indicates that a single allele was targeted in male cells and the female cells were hemizygous knockouts (Fig. 1D). Furthermore, RT-PCR analysis of a targeted female PGC line did not detect expression from the endogenous *DDX4* locus (Fig. S1). It has been reported that targeted insertion using site-specific nucleases can produce a targeted gene locus and disruption of the second chromosomal locus by NHEJ (Merkle et al., 2015). To address this concern, we sequenced the genomic

locus of the non-targeted allele in the targeted male PGC lines and did not detect any instances of indels in the second chromosomal locus (Table S1). It is possible that we did not detect any NHEJ in the second allele because bi-allelic ablation of DDX4 is lethal to male PGCs. This is unlikely as the targeted female PGC lines proliferated normally in culture (Fig. S1 and data not shown). Accordingly, precise TALEN-mediated targeting of the *DDX4* locus was achieved.

We next asked if by varying the genomic location of the right homology arm larger deletions of the *DDX4* locus could be made whilst still co-transfecting with a single TALEN pair. The 4.3 kb right homology arm was replaced with 1.5 kb arms located after exon 10 or after exon 19, the final protein encoding exon, of the *DDX4* gene. These targeting vectors would produce a genomic deletion of 10.3 kb and 30.2 kb, respectively, after correct integration into the *DDX4* locus (Fig. 2A,B; left panels). Following transfection, selection and expansion of PGCs, analysis of genomic DNA using primers located out with the homology arms revealed that the GFP-2a-puromycin transgene was precisely recombined into the endogenous *DDX4* locus and a series of deletion alleles was produced encompassing the entire locus (Fig. 2,B; right panels).

Generation of targeted G$_1$ offspring

Targeted male cells (Z^{GFP}Z) (indicated by an arrowhead, lane 10, Fig. 1D) were injected into surrogate host chicken embryos (day 2.5, stage 16HH), incubated until hatching, and raised to sexual maturity. Two host male cockerels were assayed for the presence of the GFP reporter transgene in their semen and mated to wildtype hens (Table 1). One founder male did not transmit the targeted allele to offspring whereas the second male generated 17 G$_1$ targeted offspring (6%; Fig. 3A and Table 1). Southern blot analysis of genomic DNA from the G$_1$ transgenic offspring demonstrated that the male chicks were heterozygous for the targeted allele and the female chicks were hemizygous mutant for *DDX4* (Fig. 3B). The southern blot pattern exactly replicated the pattern seen in the PGC transfections (Fig. 1D) confirming that the PGCs were targeted at a single allele.

DDX4 is required for female fertility in birds

In commercial layer hens, ovulation begins at week 18 post-hatch from a single ovary originating from the left gonad. The mature ovary contains several thousand small white follicles and 30-100 yellow or small yolky follicles (Gilbert et al., 1983). As *Ddx4* knockout female mice exhibited a normal reproductive capacity, the G_1 chickens were raised to sexual maturity with the intention to cross the females and males to generate homozygous $Z^{GFP}Z^{GFP}$ males. Unexpectedly, the seven hemizygous $Z^{GFP}W$ female G_1 chickens did not enter into lay by 29 weeks post-hatch (PH). A morphological examination of the ovaries from these hens revealed that no white or yellow follicles were visible and no primary or secondary follicles were detected in sections (Fig. 4A and Fig. S2). To examine the earlier stages of ovarian development a $Z^{GFP}Z$ heterozygote male was mated to wildtype ZW hens and ovaries of age-matched $Z^{GFP}W$ and ZW hatchlings were examined post-hatch. In the hatchlings, primary and secondary follicles surrounded by layered granulosa cells were present at two and four weeks PH (n = 6; ZW) whereas no follicles were detected in the ovaries of the $Z^{GFP}W$ chicks (n = 6; Figs 4B,C). Similarly, immunostaining for GFP and germ cell markers of mature oocytes, p63 and MLH1, did not reveal any germ cells in the ovary at week two PH (Fig. 4D-G).

To identify whether the defect in oogenesis was due to ablation of the germ cell lineage, we examined early embryonic stages of the $Z^{GFP}W$ embryos. DDX4 protein marks the germ cell lineage at cleavage stages of embryonic development and *DDX4* RNA is expressed during PGC migration to the gonad and at all subsequent developmental stages (Tsunekawa et al., 2000). We found that GFP$^+$ cells were present in $Z^{GFP}W$ gonads at day 6 and day 9 of incubation (Fig. S3A,B). These cells expressed the germ cell marker, SSEA1. Immunostaining with CVH confirmed the lack of DDX4 protein in day 9 female PGCs of $Z^{GFP}W$ embryos (Fig. S3B). We examined the number of proliferative cells in the gonad of day 10.5 embryos to determine if proliferation was compromised in the developing gonad in the absence of DDX4. We observed that there was a significant reduction in EdU$^+$ PGCs in the developing cortex of $Z^{GFP}W$ gonads (Fig. S3C). These results show that germ cells initially form in $Z^{GFP}W$ chicken embryos and are lost at later developmental stages.

To determine if the $Z^{GFP}W$ germ cells entered meiosis, several meiotic markers were examined in the developing ovary. PGCs in the female gonad enter meiosis by day 16 of embryonic development and most oocytes reach the diplotene stage of meiosis I by day 7 PH (Smith et al., 2008; del Priore and Pigozzi, 2012). In control embryonic day 17 ZW gonads, germ cells correctly express the meiotic markers SCP3, γH2AX, and MLH1 (Fig. S4). In contrast, SCP3 was not detected at this stage and germ cell number was reduced in the $Z^{GFP}W$ ovary. By embryonic day 19, $Z^{GFP}W$ germ cells expressed SCP3 at levels comparable to wildtype ZW germ cells, although visibly less germ cells were present in the cortex of $Z^{GFP}W$ gonads (Fig. S4). The widespread expression of MLH1 indicated many germ cells in both $Z^{GFP}W$ and ZW gonads had reached the pachytene stage of meiosis I. Expression of SCP1 was also detected in embryonic day 19 $Z^{GFP}W$ embryos (Fig. S4). To determine if the remaining $Z^{GFP}W$ germ cells progressed to later stages of meiosis, chicks were examined at three days PH. In chickens, the majority of germ cells have entered the pachytene stage of meiosis at day 3 PH (Hughes, 1963; del Priore and Pigozzi, 2012). In control ZW day 3 hatchlings, SCP3$^+$, MLH1$^+$ germ cells were present in the cortex of the ovary and SCP3 was expressed in a punctate manner indicative of the pachytene stage of meiosis (Fig. S4) (Guioli et al., 2012). In contrast, the majority of the GFP$^+$ cells were located in the medulla of the $Z^{GFP}W$ day 3 PH ovary. The limited number of GFP+ cells found in the cortex were MLH1$^+$ but very few expressed SCP3. These data are in agreement with a defect in progressing beyond the pachytene stage of meiosis leading to a post-hatch loss of germ cells in the $Z^{GFP}W$ ovary.

Discussion

These results demonstrate that TALEN-stimulated HDR in germ cells is highly efficient and opens future avenues for investigation of gene function in birds and for the introduction of production traits. Large genomic deletions have been achieved using two site-specific nucleases or CRISPR vectors but to our knowledge, this is the first large genetic deletion produced using a single nuclease pair. Genetic deletions through altered placement of homology arms will be useful to create a series of genetic deletions at loci of interest. CRISPR-stimulated HDR in chicken germ cells

has been reported but the recombination efficiency was much lower than reported here (Dimitrov et al., 2016). As *DDX4* is expressed in PGCs, it is possible that this genomic locus may be highly accessible to site-specific nucleases and amenable to gene targeting. Additionally, the cell culture medium used in this report supports rapid cell proliferation (Whyte et al., 2015) that may lead to a much higher frequency of HDR. We did not observe a phenotype in cultured female targeted PGCs or at early embryonic stages in $Z^{GFP}W$ embryos. Early PGCs express the dead box genes *DDX43* and *DDX25* (Jean et al., 2015). It is possible that these proteins replace *DDX4* at early developmental stages.

The precise role of played by DDX4 in meiosis is still unknown. The mouse *Ddx4* knockout led to male sterility; male mouse PGCs enter meiosis but did not express diplotene markers and undergo apoptosis. Proliferation of $Ddx4^{-/-}$ PGCs was also severely compromised in the early mouse gonad similar to what was observed here in the forming ovary of $Z^{GFP}W$ embryos (Tanaka et al., 2000). *Ddx4* is thought to function in amplifying translation of a number of proteins needed for meiotic progression and the assembly of cytoplasmic granules in germ cells which are potential RNA processing centres (Aravin et al., 2009; Kuramochi-Miyagawa et al., 2010). Future experiments will address the function of DDX4 in chicken germ cell meiosis.

Materials and Methods

PGC culture

PGC line derivation and culture were carried out as described in (Whyte et al., 2015). Briefly, 1 ul of blood isolated from a stage 16 HH embryo was placed in culture medium containing 1x B-27 supplement, 0.15 mM $CaCl_2$, 2.0 mM GlutaMax, 1x NEAA, 0.1 mM β-mercaptoethanol, 1x nucleosides, 1.2 mM pyruvate, 0.2% ovalbumin (Sigma), 0.01% sodium heparin (Sigma). Activin A, 25 ng/ml (Peprotech), FGF2, 4 ng/ml (R&D Biosystems), 5 µg/ml ovotransferrin (Sigma) in Avian Knockout DMEM (250 osmoles/litre, 12.0 mM glucose, calcium chloride free (Thermo Fisher Scientific, Custom modification of Knockout DMEM). 0.2% chicken serum (Biosera)

was added to this medium to produce FAOTcs medium. A male and a female PGC line were derived in FAOTcs medium, expanded to 250,000 cells in five weeks before use in targeting experiments.

TALEN design and construction

All TALENs were designed using the TALE-NT software and assembled using methods described in (Cermak et al., 2011), et al. (2011). Design and construction of ggVASAe1.1 is described in (Carlson et al., 2012) using the pC-GoldyTALEN (Addgene ID 38143). The TALEN pair creating a cleavage site 15 bp 5' to the ATG of DDX4 were designed (DDX1.1 Targeting vector) with the following binding sites: left monomer: (sense) GCTAACGTGCTCCTGGTCCT); right monomer: (sense) ATTCGCTATGGAGGAGG). A 2.9kb genomic fragment upstream of exon 2 of the DDX gene was PCR amplified from ISA brown genomic DNA and cloned upstream of a GFP-2A-puromycin-polyA expression cassette (Hockemeyer et al., 2011) by converting the endogenous ATG to an Nco1 site (outer primers CTGGTAGAGAGCATTACAAAAGTC; GTGTCCCAGTCCTCCTCCATAG; inner Nco1 primer AACCATGGCGAATGCCAGCAGCCCA). A 4.3kb downstream PCR fragment containing exons 4-6 was cloned into a BAMH1 site downstream of the polyA site to create pddx4-GFP-polyA-exon4 using nested primers (outer primers CTCCTTGGCCCCATTAACAGA GTTTTGTGCCATGACCACTG; inner primers GGGGCCCAGAAGTTCTCCTTA TTGGGCCCAAATCCACGGTGCAATATCC). This right arm was replaced by digesting with BamH1 and the following right arms were used: a 1.5 kb PCR fragment containing intron 10 (pddx4-GFP-polyA-intron10), (primers TAGTTGGATGCCTCAGACTTCA, ATTGCAAGTGGAGCTTCAAGA) and a 1.5 kb PCR fragment downstream of exon 19 (pddx4-GFP-polyA-3pUTR) (primers GAAGGCAAAAGCCATTTTCA, CCCTTCTAAACCCTGCAATTC) using Phusion HF polymerase (New England Biolabs).

PGC transfection and electroporation

1 µg of the TALEN vector pair 1.1 (0.5 µg of left and right) and 1 µg of the targeting vectors were co-transfected into PGCs using DIMRIE transfection reagent as previously described (Macdonald et al., 2012a). Briefly, 100,000 PGCs were washed in Optimem I (Thermo Fisher Scientific), mixed with the DNA and transfection

reagent and transfected in suspension for six hours. PGCs were then centrifuged and re-suspended in Facs medium. For electroporation, 100,000 PGCs were centrifuged and re-suspended in 10 µl of solution R containing 1ug of DNA (0.5 µg of the TALEN vector pair 1.1 and 0.5 µg of the targeting vector) and electroporated using a Neon electroporator (Thermo Fisher Scientific); 850 volts, 50 msec pulse. To measure targeting efficiencies, PGCs were immediately selected 24 hours post-transfection with 0.6 µg/ml puromycin for 48 hours, then washed to remove all puromycin and further cultured for two weeks to eliminate transient GFP florescence. To select stably transfected cells, PGCs were selected at four days post-transfection using 0.3 µg/ml puromycin treatment over a two week period. PGCs from each individual transfection were then expanded in culture for 29 days to 800,000 cells and cryopreserved using Avian KO-DMEM/B-27 supplement containing 5%DMSO/4% chicken serum and frozen for 9 months before use in germ line transmission experiments. Genomic DNA was isolated from each individual transfection and used for Southern blot analysis.

Immunohistochemistry

Tissues were fixed in formalin for paraffin sections followed by Haemotoxylin/Eosin staining or cryo-embedded and processed for immunofluorescence (Whyte et al., 2015). The number of follicles per field for 2 week post-hatch ovaries was determined by counting one microscope field per slide for 4 slides from 4 different ovaries for each genotype. Samples were incubated with the primary antibody in 5% goat serum overnight at 4°C. Details of antibodies used are provided in the supplementary Materials and Methods.

Flow cytometry

For flow cytometry analysis, transfected PGCs were transiently selected with puromycin at 24hours post-transfection to enrich for transfected cells. PGCs were treated for 48 hours then washed to remove puromycin. PGCs were cultured for an additional three weeks then analysed for GFP fluorescence using a BD FACSAria II machine (BD Biosciences) to identify stable integrated cells.

Germline transmission

For germline transmission, Y25 male-targeted cells (Novagen Brown) were thawed after storage at -150°C for 9 months and cultured for 4-8 days before injection into stage 16 HH surrogate hosts embryos in windowed eggs (Whyte et al., 2015). 3000-5000 PGCs were injected into the dorsal aorta, the shells were resealed with parafilm and the eggs were incubated at 37.7 °C until hatching (McGrew et al., 2004b; Nakamura et al., 2008). Four injection experiments were carried out. PCR screening for the GFP transgene in the semen of two founder male cockerels was performed and these were then bred to wildtype hens. Offspring were screen by PCR for the presence of the GFP transgene (McGrew et al., 2004a). Genomic DNA was isolated from the blood of GFP$^+$ positive G_1 offspring and used for Southern blot analysis. Animal experiments were conducted under UK Home Office license.

Southern blot analysis

5 µg of genomic DNA isolated from blood samples or stably transfected PGCs (~1,000,000 cells) using a Flexigene kit (Quiagen). DNA was digested overnight using Mfe1. The DNA digests were resolved by gel electrophoresis and transferred via capillary action to Hybond N membrane (GE Healthcare). A GFP fragment (0.8kb) or a 0.6kb *DDX4* genomic DNA fragment (amplified using primers: GACAAGCCATCACATACAAAGC, AAGGAAGCTGGGAGCTCTTC), were labelled with α-^{32}P-dCTP using a DIG High Prime DNA Labelling and Detection Kit II (Roche) and used to hybridise the Southern blot.

To assay integration into the ddx4 locus by PCR, the following nested primers were used for the common left arm: outer primers (CAGCACTGTTAAAGGGCACA, AAGTCGTGCTGCTTCATGTG); inner primers (GCGCGCTTTGACATATTTTT, GGTCACGAGGGTGGGCCAG); 11kb right arm (GCCTGAAGAACGAGATCAGC, TCCACTGCCATATGAGGACA); 20kb right arm (GCCTGAAGAACGAGATCAGC, GGGGTTGGACTTAATCTCTGG)

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Competing interests

S.C.F. and D.F.C. are full-time employees of Recombinetics, Inc.

Author contributions

M.J.M. and S.C.F. conceived the experiments. L.T., D.F.C., A.S., S.N., and M.J.M. designed and performed experiments. L.T., D.F.C., S.N., and M.J.M. wrote the paper; all authors read and approved the paper.

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Figures
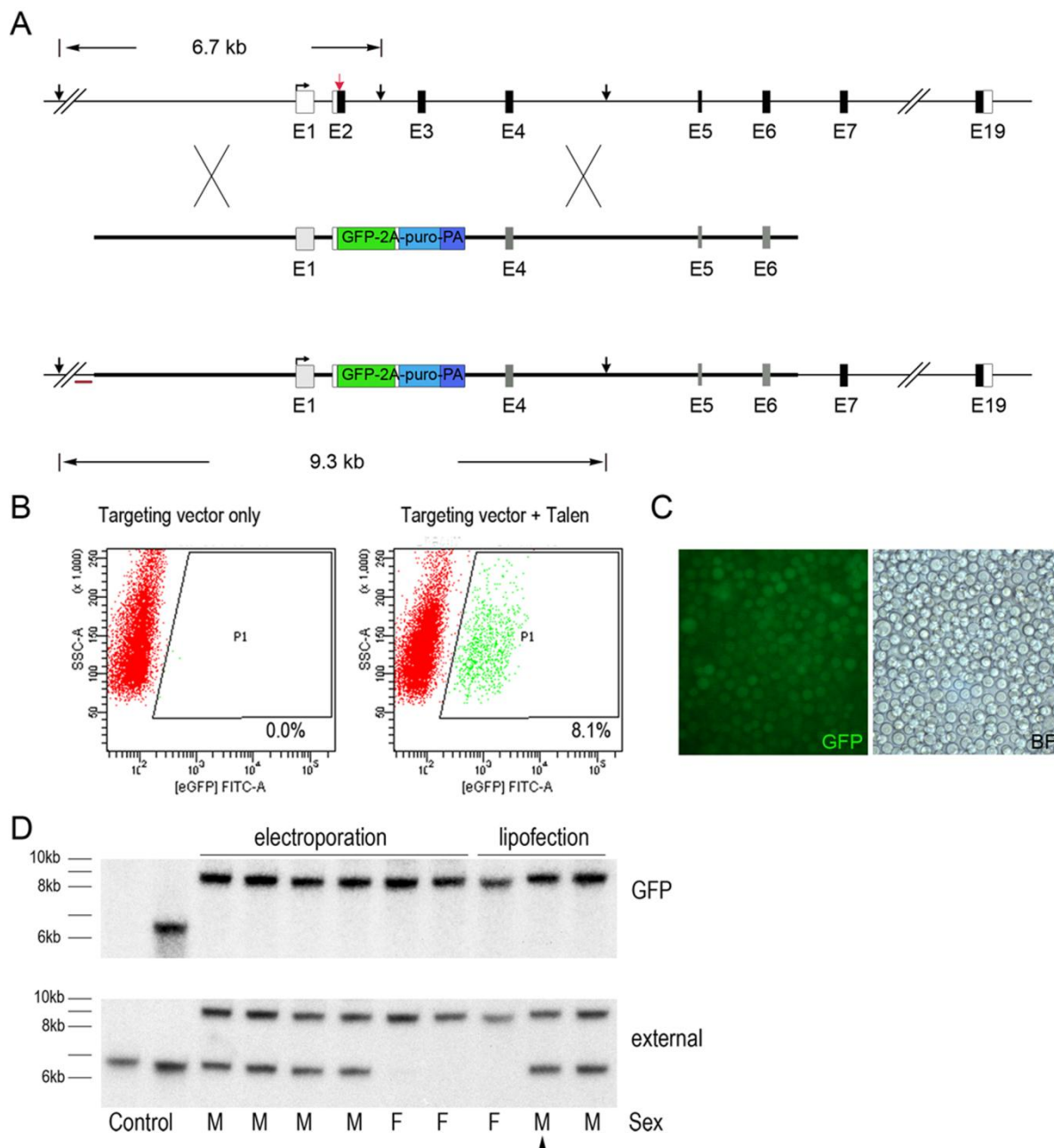
Fig. 1. Targeting of the *DDX4* locus using TALENs.

(A) Schematic overview of targeting strategy to generate a knockout/knock-in ddx4 allele. The TALEN pair cleavage site is indicated, red arrow. Protein coding exons are indicated in black boxes. Black arrows, Mfe1 sites.

(B) Targeting efficiencies of cultured PGCs. PGCs (male) were transfected with the targeting vector with or without the TALEN constructs, selected with puromycin for two days to enrich for transfected cells, cultured for two weeks and analysed by flow cytometry. The data represent one of two independent experiments with similar results.

(C) Targeted GFP$^+$ PGCs in culture after selection with puromycin.

(D) Southern blot analysis of GFP-puro targeted allele. External probe; Expected fragment size: wildtype, 6.7 kb; targeted; 11.7. Internal GFP probe; Expected fragment size, targeted, 11.7 kb. The genotype of the cells is indicated by M (male) and F (female). Arrowhead, cell line used to generate targeted chicken.
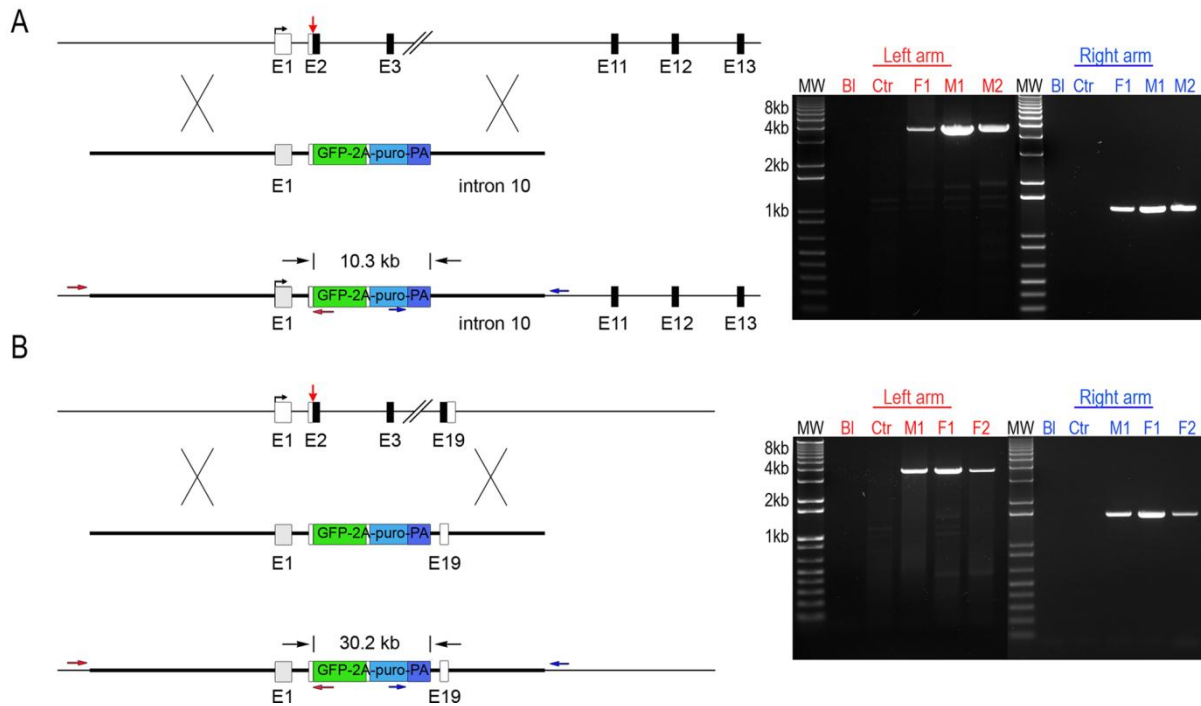
Fig. 2. Large genomic deletions of the *DDX4* locus using alternative homology arms.

(A,B; left panels) Schematic of strategy to generate larger deletions of the *DDX4* locus. The TALEN dimer is indicated by the red arrow and the positions of the right targeting arms are shown. The 5' primers sets are shown in red and the 3' primer sets in blue.

(A, right panel) PCR genotype analysis of the *DDX4* targeted 9 exon deletion using external and internal amplification primers. Expected product size: left arm 3.6 kb, right arm 1.8 kb. Bl, blank; Ctr, control non-transgenic DNA; M1, M2, male cell line; F1, F2, female cell line.

(B, right panel) PCR genotype analysis of the *DDX4* targeted 18 exon deletion using external and internal amplification primers. Expected product size: left arm 3.6 kb, right arm 1.6 kb. Bl, blank; Ctr, control non-transgenic DNA; M1, M2, male cell line; F1, F2, female cell line.
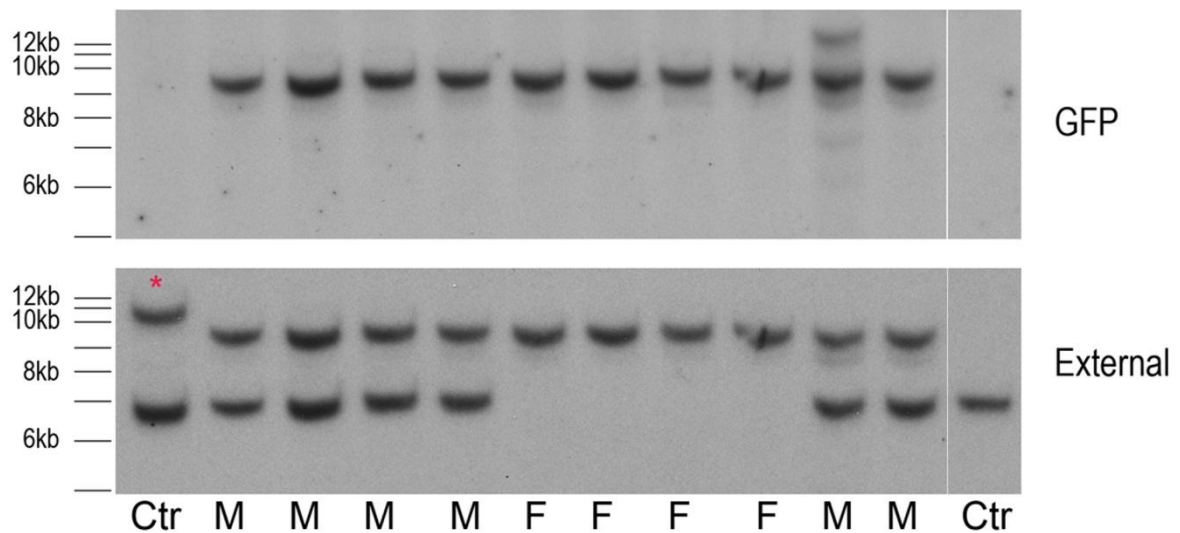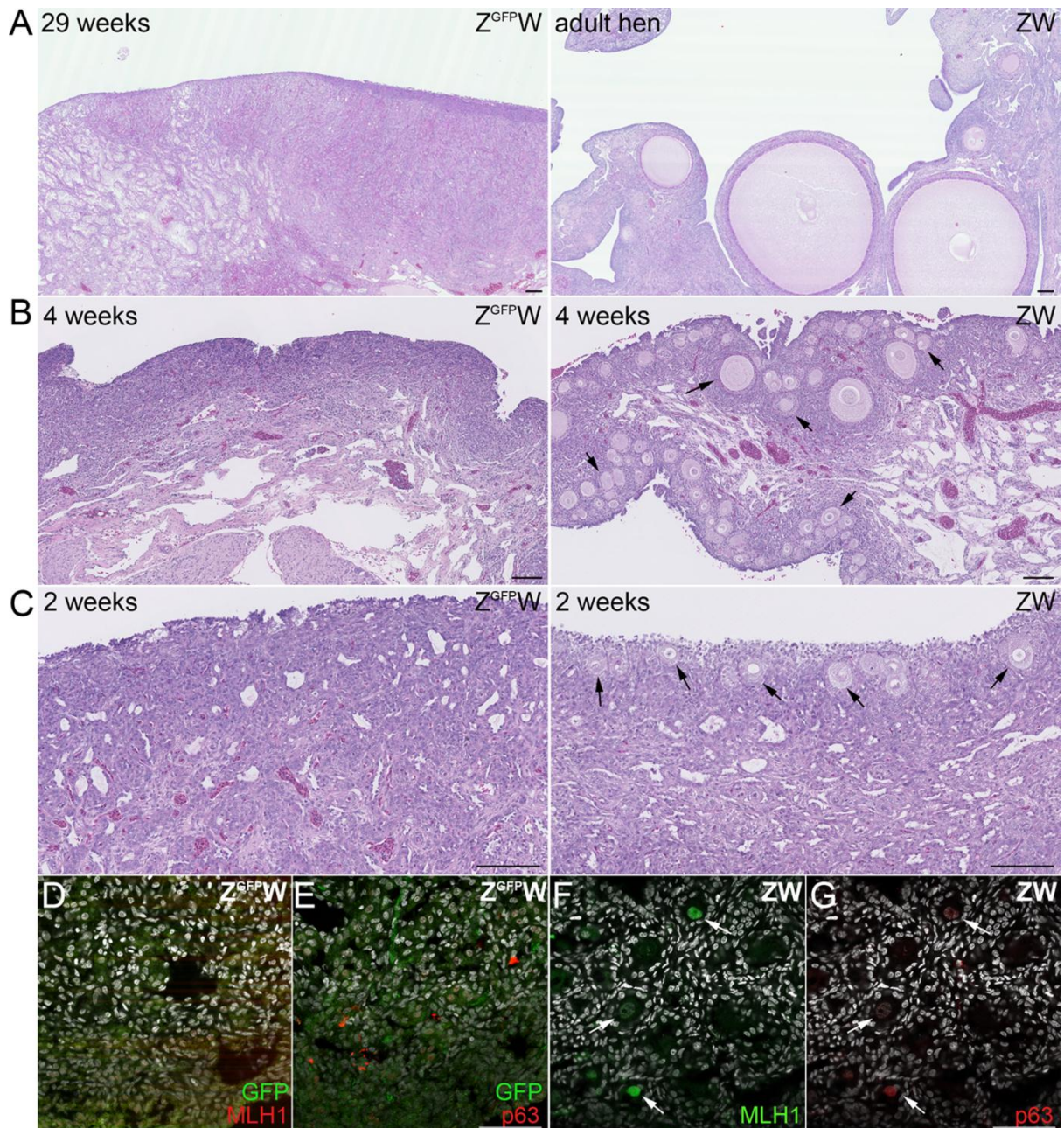
Fig. 3. Targeted knockout offspring produced from *DDX4* targeted PGCs.

(A) Targeted male and female G_1 chicks.

(B) Southern blot of control and individual G_1 offspring. Fragment sizes are equivalent to those in Fig. 1. *, larger DNA fragment generated by restriction fragment length polymorphism in the control DNA sample.

Fig. 4. No follicles are present in the cortex of post-hatch Z^{GFP}W hens.

(A-C) Haematoxylin and Eosin (H&E) staining of representative post-hatch $Z^{GFP}W$ and ZW hens. Arrows indicate developing follicles. (c) $Z^{GFP}W$, 0 follicles/field; ZW, 26 follicles/field; average of 10 fields from 4 ovaries/genotype. Bar, 100µm.

(D-G) Immuno-staining of 2 week post-hatch follicles for GFP and the germ cell meiotic markers p63 and MLH1. Nuclear stain, white. Arrows indicate developing follicles. Bar, 50µm.

Table 1 Germline transmission frequency of donor Z^{GFP}Z male PGCs in surrogate host chickens.

| Founder Birds ♂	Eggs set	Chicks hatched (%)	% genome equivalents in semen	Ddx$^{+/-}$ offspring (% transmission)
Ddx 1-3	311	268 (86%)	10%	17 (6%)
Ddx 4-12	143	104 (73%)	5%	0 (0%)

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WT   CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
Y21  CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
Y21  CGTCAGGGAGGGGCGGGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
Y21  CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
Y22  CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
Y22  CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
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Y26  CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
Y26  CGTCAGGGCGGGGGGTGCTGCTAACGTGCTCCTGGTCCTGCAGGTGGGCTGCTGGCATTCGCTATGGAGGAGGACTGGGACACGGAGCTGGA
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## Table S1. Sequence of the non-targeted allele in Z^GFP^Z PGC lines

The six independently targeted male PGCs lines show in Fig 1D were PCR amplified and sequenced at the non-targeted DDX4 allele to detect potential indel mutations. Red; TALEN binding sites.
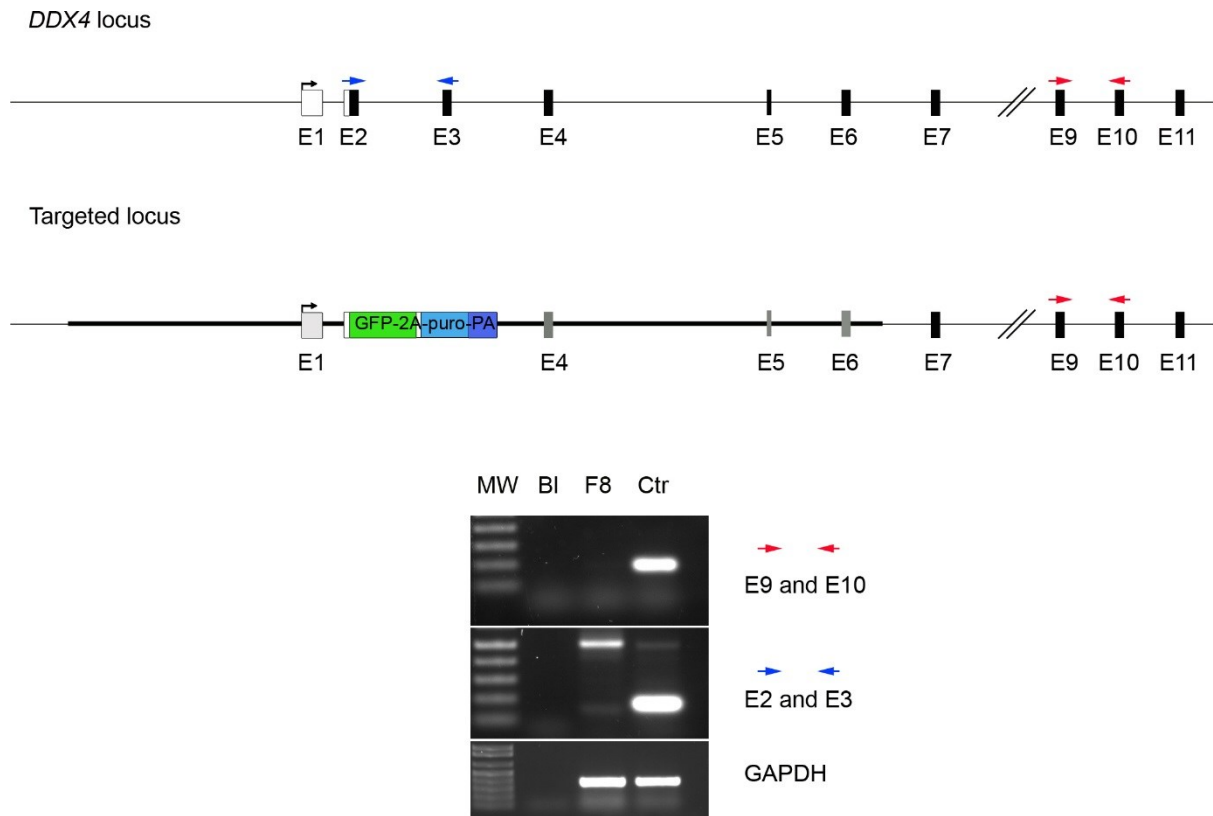
Figure S1. DDX4 expression in targeted female PGCs

RT-PCR was carried out on cDNA from cultured chicken cells lines. Bl, Blank, F8, *DDX4* targeted female cell line 8; Ctr, untargeted female cell line 8.
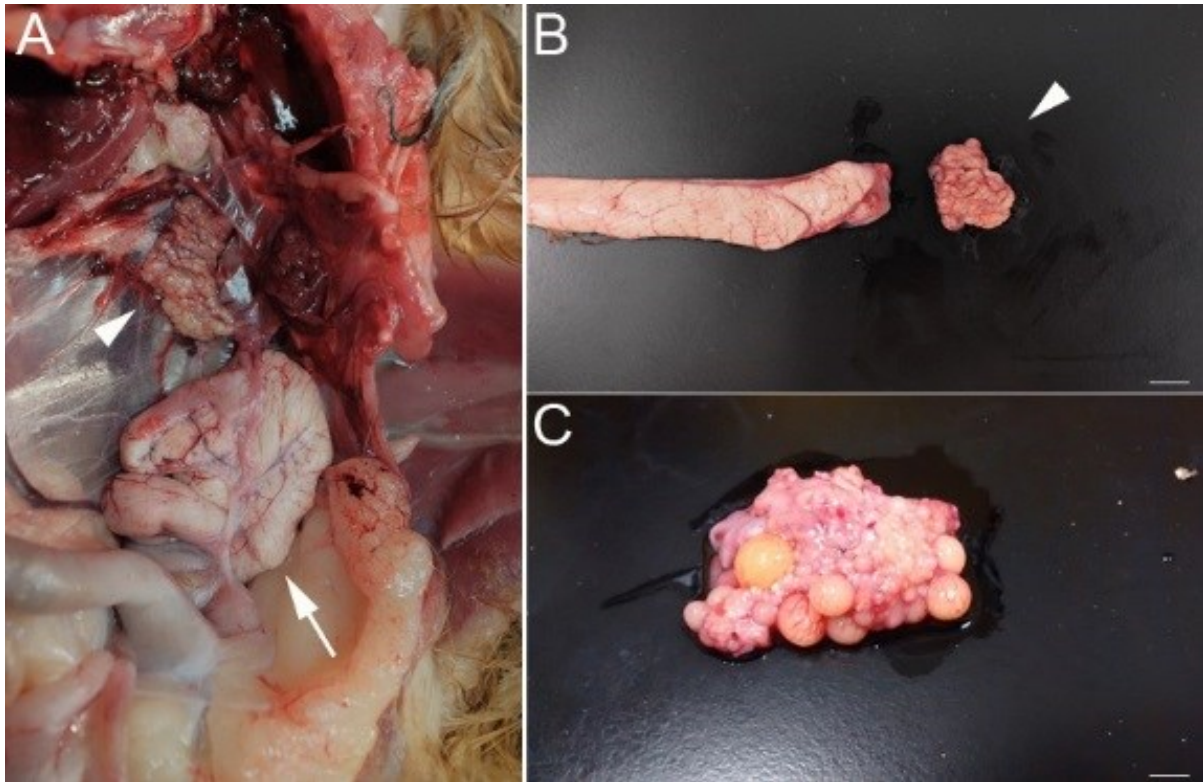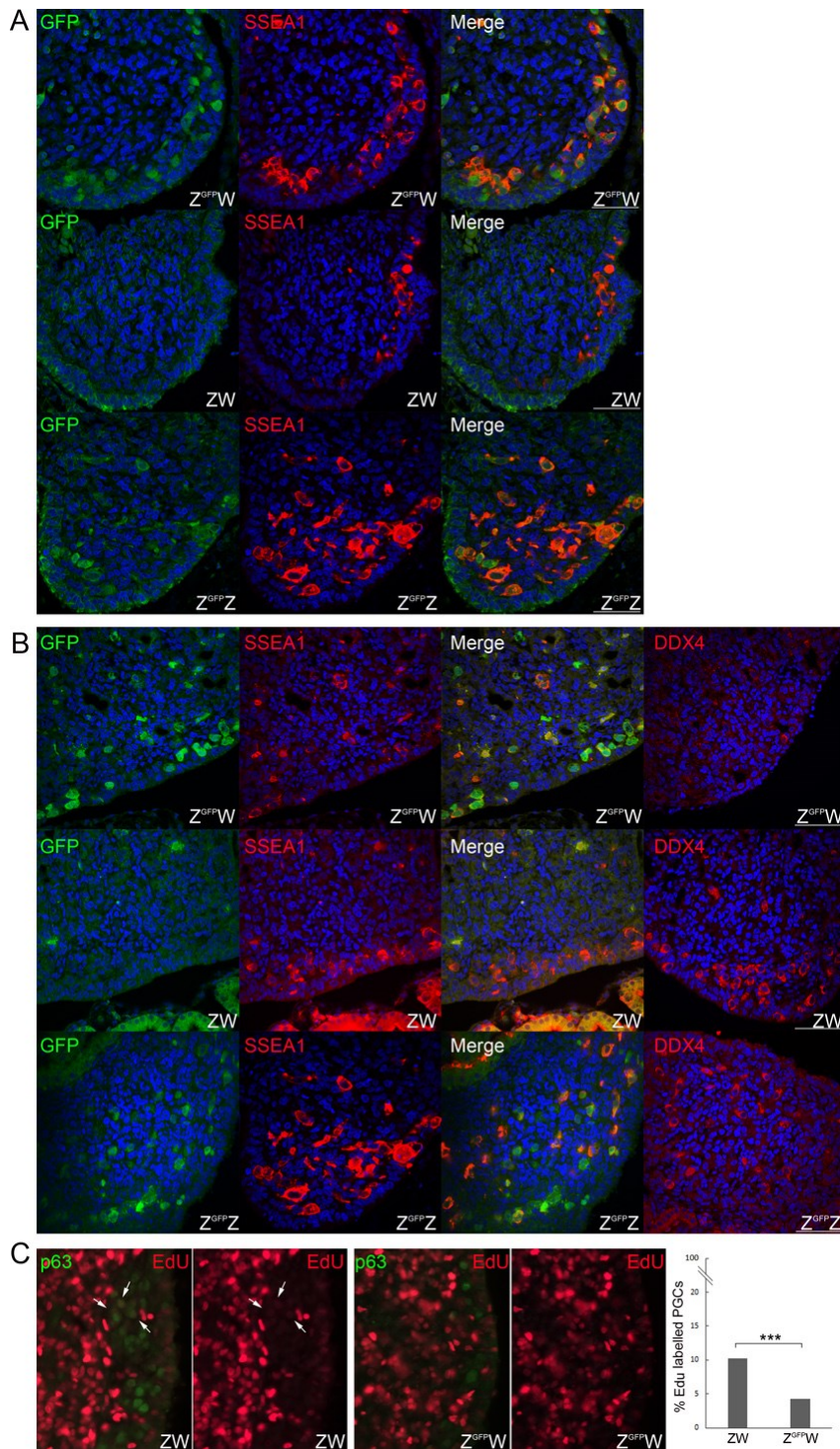
Figure S2. Follicles are absent in Z^{GFP}W hens.

(a) Oviduct (arrow) and immature ovary (arrowhead) in a 29 week old Z^{GFP}W hen.

(b) Ovary (arrowhead) of a 29 week old Z^{GFP}W hen. Bar, 1 cm.

(c) Ovary containing developed follicles of a ZW control hen. Bar, 1 cm.

Figure S3. PGCs are present in Z^{GFP}W embryos

Analysis of GFP fluorescence and immunostaining for SSEA1and DDX4 at A) day 6 and B) day 9 of incubation. Blue, nuclear stain. Scale bar, 100 µm.

C) Proliferation of PGCs in day 10.5 gonads using EdU labelling. Representative sections from ZW and Z^{GFP}W embryos. PGCs were identified by immunostaining for p63 and were scored as EdU$^+$ or EdU$^-$. ***, $p < 0.001$ using a Fisher's exact test two-tailed P value.
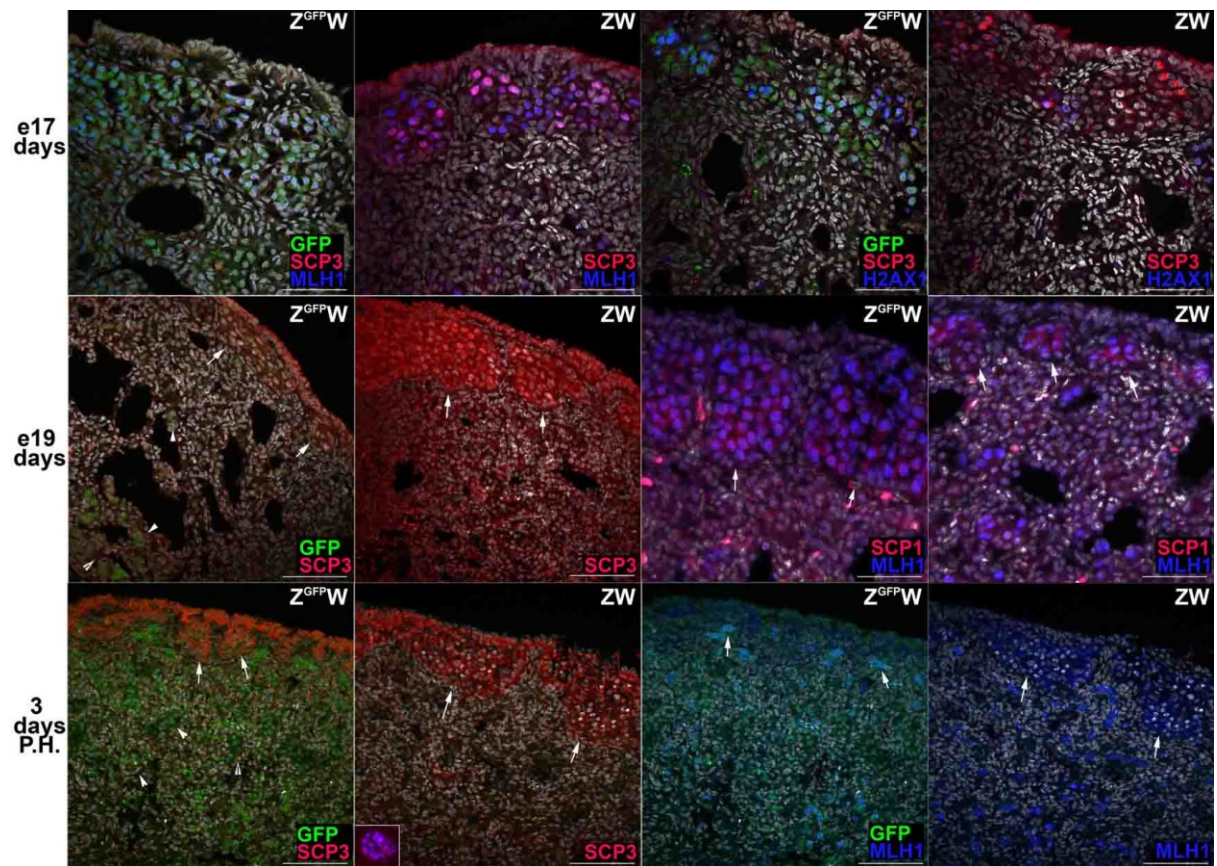
Figure S3. Z^{GFP}W germ cells fail to progress through meiosis and are lost postnatally.

Immunostaining of SCP3, SCP1, γH2AX, and MLH1 and GFP fluorescence in embryonic day 17 and 19 (e17 and e19) embryos and 3 days PH chicks. Arrowheads indicate germ cells located in the medulla of the ovary, arrows indicate germ cells located in the cortex of the ovary. Insert in 3 days PH ZW image is the enlargement of a single cell showing punctate staining pattern of SCP3. Nuclear stain, white. Bar, 50 um.

Supplementary materials and methods:

Immunohistochemistry

Primary antibodies were: rabbit anti-DDX4 antibody (kind gift from Craig Smith (Monash University, Melbourne), SCP1 (Novus, rabbit), SCP3 (Abcam, rabbit), MLH1 (BD, mouse), γH2AX (Millipore, mouse), p65 (Abcam, rabbit). Sections were washed with PBT for 30 min, and incubated with secondary antibodies conjugated with Alexa-Fluor 488 555, or 647 (Thermo Fisher Scientific) for 1 h at room temperature. Samples were washed PBT for 30 min, and stained with Hoechst (Sigma) to visualize nuclei. Cells were mounted under coverslips in PBS and cryosections were mounted in Diamond Gold (Thermo Fisher Scientific) and visualized using a Zeiss LSM 710 inverted confocal microscope. Images were captured using Zen Black software (Zeiss). For EdU staining 0.5 ml of 0.4 mM EdU solution was injected near the amniotic sac of day 10.5 embryos. The eggs were sealed, incubated for 6 hours and the embryos were fixed and processed for immunohistochemistry. The Click-iT EdU Alexa Fluor 555 kit (Thermo Fisher Scientific) was used to stain and PGCs were then co-stained using the p65 antibody. Three embryos for each genotype were analyzed. Images were taken on a Leica DMLB microscope.

RT PCR analysis

Total RNA was isolated from control and targeted female PGCs using the RNeasy Mini Kit (Qiagen). Total RNA (500 ng) was reverse-transcribed. PCR conditions were 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30 cycles. PCR conditions for exon 2 and 3 reactions were 94 °C for 5 min, 94°C for 30 s, 50 °C for 30 s, and 72 °C for 1 min for 30 cycles Reaction products were resolved on 1% agarose gels and visualised using a transilluminator. Primer sequences were:

GAPDH: fwd, CCTCTCTGGCAAAGTCCAAG   rev, CATCTGCCCATTTGATGTTG

Exon2&3: fwd, ATGGAGGAGGACTGGGACA   rev, AGCCAAAGAAGGGGCTGT

Exon9&10: fwd, TCCATCTTTGCATGTTATCAGTCAGG rev, AATCCCGCCCTGCTTGTATAACAG