SUPPLIMENTARY INFORMATION

for on-line publication

Legends to Supplimentary Figures

Suppl. Figure 1. Conditionally targeting the *Oct4* locus. A two-step *loxP*-flanking ('floxing') strategy (Gu et al. 1994; *Science* **265:** 103-106) was adopted to generate mutant ES cells carrying a functional *flox* allele of *Oct4*. The strategy was to convert a single *flox* allele by Cre recombinase into a null (*Δ*) allele in mice derived from these ES cells. In the first step, a DNA construct containing the 34-bp α *loxP* site (filled arrow) just upstream of the 200-bp essential *Oct4*promoter (oval) and a *neo* cassette flanked by the identical, co-directional β and γ *loxP*s were introduced into the first intron of the Oct4 gene by homologous recombination in ES cells. To avoid disrupting potential *cis*-regulatory elements, the indicated insertions were made in Oct4 promoter area that is divergent between various species. (*A*) Schematic representation of the targeting DNA construct, wild-type (+), *floxNeo*, *flox*, and *Δ* alleles of *Oct4* gene. Additionally shown *loxP* sites (filled arrowheads, α through γ), gene exons (filled rectangles, 1 through 5), and positions of the probe and primers used for genotyping in *C* and *D*. In the second step, the successfully targeted *+/floxNeo* ES clones were subjected to a transient, balanced expression of Cre recombinase to generate the *flox* and *Δ* alleles. The *flox* allele was obtained by partial (β to γ *loxP*s), and the *Δ* allele was obtained by complete (α to γ *loxP*s) excision in the *floxNeo* allele (A). ES cell clones with an excision between α and β *loxP*s were not used in further analyses. (*B)* Northern blotting of the poly A^+ RNA from the wild-type (+/+) and heterozygous mutant ES cells probed with labeled Oct4 cDNA. The 1.6-kb band corresponds to the size of the Oct4 transcript from the + and *flox* alleles. The *Δ* allele did not generate any transcripts. Due to the excision between the α and β *loxP* sites, the transcript should have been smaller than 1.6 kb. The Northern blot results

suggest that neither allele was hypomorphic. *(C)* Southern blot analysis of EcoRI-digested genomic DNA from ES cells or mice. The probe indicated in *A* detects the +, *floxNeo*, and *Δ Oct4* alleles as 12-, 7.1-, and 10.8-kb fragments, respectively. Note that the Southern strategy does not allow us to distinguish the + and *flox*. (*D)* PCR genotyping of ES cells, embryos, and mice. Primer pair A detects both + and *flox Oct4* alleles, amplifying 498- and 532-bp fragments (498+34 bp of the *loxP*), respectively. Similarly, pair B is diagnostic for the βγ *loxP,* amplifying 415- and 449-bp fragments from the + and *flox* alleles, respectively. Pair C produces a 245-bp fragment from the *Δ* allele. Notice that the *flox/flox* and *flox/Δ* genotypes were obtained only in mice through breeding of the *flox* and $TNAP^{Cre}$ mice (see Suppl. Fig. 1). The *flox/+* ES cells were used to derive a mouse line through blastocyst injection. We were able to breed and maintain these mutant mice in a homozygous state for more than 15 generations. Thus, the full function of the *flox* allele and lack of function of the *Δ* allele (see Suppl. Fig. 2) suggested that the *flox* mice would be a suitable substrate for the Cre-mediated conditional knockout of *Oct4*.

Methods

Targeting construct. The *ApaI-BamHI* ~6.1-kb fragment of the *Oct4* gene was subcloned into pBS-KS and used to insert a double-stranded oligonucleotide containing a *loxP* site at the *AatII* and *loxP*-flanked ('floxed') *Tk*/*Py-neo* cassette at the *ApaL1* site in the first *Oct4* intron. The cassette was excised from the pNPK-CreAR3 plasmid that had been modified by inserting *ApaL1*/*loxP* site-containing oligonucleotides around the *Tk*/*Py-neo* sequence. The targeting DNA fragment (~35μg) was excised from pGOF6.1-floxNeo (construct #17-2) with ApaI and NotI and electroporated (240V, 500μF; BioRad's Gene Pulser) into ES cells (10^7). After 10-12 days of selection in the presence

of G418 (350 μg/ml, Gibco-Invitrogen), resistant clones were screened for homologous recombination by Southern blotting.

ES cells. E14 ES cells were routinely cultured on mitomycin-inactivated mouse embryonic fibroblast (MEF) cell layer in DMEM supplemented with ESGRO (1000 units/ml, Chemicon), 15% fetal calf serum (HyClone), 100 μM non-essential amino acids, 100 μM β-ME, 2mM L-glutamine, and 50 μg/ml penicillin/streptomycin (Gibco).

Genotyping. Southern blotting of EcoRI-digested genomic DNA from ES cells was performed using the NcoI-NcoI ~1-kb Oct4 genomic fragment as a probe. The presence of the α loxP was further verified by PCR using the pair A primers (GOF-AatII: 5'-aactggtttgtgaggtgtccg-3', GOF-HindIII: 5'-tcgtatgcgggcggacatg-3'). Two successfully targeted ES clones (*Oct4*$^{+/floxNeo}$) were further expanded and subjected to the next round of electroporation, with 5 μg of the circular pHC-Cre. The β-γloxP (flox allele) and α-γloxP (Δ allele) deletion events were detected using the primer pair B (GOF-D1: 5'-ttgttactgaagaggttgggtgtgactgg-3', GOF-R1: 5'-ggggactcctgctacaacaatcgctaag-3') and pair C (GOF-AatII: 5'-aactggtttgtgaggtgtccg-3', GOF-ApaL1: 5'-gtatccactcgcaccttgttc-3') primers, respectively. The *TNAP*^{Cre} allele was detected by PCR using the genCre-D: 5'-ccacgaccaagtgacagcaatg-3' and genCre-R: 5'-cagagacggaaatccatcgctc-3' primers, producing a 373-bp amplicon. Genomic DNA was extracted from ES cells, embryo heads and tail biopsies in buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2% SDS, 200 mM NaCl, and freshly added proteinase K (200 μg/ml) at 55^{0}C for 6-15 hrs.

Mice. Two clones of Oct4$^{+/flox}$ ES cells were injected into C57Bl6 blastocysts. Chimeric males were tested first for germline transmission by backcrossing them to C57Bl6 females. A chimera that incorporated the mutation to the germline was further mated to

the 129/Ola mouse strain, thus retaining the $Oct4^{flox}$ allele on pure genetic background. The $TNAP^{Cre}$ mice (Lomeli et al. 2000) were of outbred 129×C57Bl6 background. The noon of the day of plug was taken as 0.5 dpc for the timing of developmental stages. ICSI was performed as previously reported (Boiani et al. 2002). Animals were maintained and used for experiments according to IACUC guidelines at the University of Pennsylvania and Max-Planck Gesellschaft.

Suppl. Figure 2. Mating strategy to generate *Oct4* deficiency in PGCs. The $Oct4^{flox}$ mice were bred to homozygosity mated to $TNAP^{Cre}$ mice (mating #1) and yielded offspring with $Oct4^{flox/+};TNAP^{Cre/+}$ genotype in somatic tissues and a Δ allele in the germline (due to Cre-mediated conversion). This conversion was expected to have limited efficiency (Lomeli et al. 2000). Because we aimed to have as many Δ/Δ progeny as theoretically possible (25%), we introduced the Δ allele into all cells of the offspring, including their germ cells (mating #2). In a parallel mating (#3), the $Oct4^{\Delta/+};TNAP^{Cre/+}$ mice obtained by the previous mating were inter-crossed, producing normal heterozygous Δ offspring. This observation is consistent with the normal viability and fertility of $Oct4^{\beta\text{-}geo}$ (null allele) heterozygous embryos and mice previously reported (Nichols et al. 1998). Homozygous Δ animals have never been found during these matings, likely due to the null function of the Δ allele and subsequent loss of embryos at the peri-implantation stage, as in the $Oct4^{\beta\text{-}geo}$ homozygotes (Nichols et al. 1998). In the final mating (#4), $Oct4^{\Delta/+};TNAP^{Cre/+}$ and $Oct4^{flox/flox}$ mice were crossed, producing one quarter of progeny with $Oct4^{flox/\Delta};TNAP^{+/Cre}$ genotype in the soma and as anticipated, the conversion of the remaining *flox* allele into Δ

and subsequent loss of Oct4 function in PGCs. The phenotype of this offspring, referred to hereafter as *Δ/Δ*, was analyzed in detail and compared to the control $Oct4^{+/\Delta};TNAP^{+/Cre}$ and $Oct4^{flox/\Delta}TNAP^{+/+}$ littermates (*Δ*/+). These two groups are in bold frame boxes. Open boxes indicate offspring with genotypes that were not used for further matings or analysis. Embryos and adult animals were genotyped as described in Suppl. Fig. 1C,D.

Suppl. Figure 3. FACS analysis of genital ridges from individual 10 dpc embryos. (*A*) Intact cells were gated by forward and side scatter (G1) for subsequent antibody detection. (*B*) The cells with SSEA1 antibody staining intensity greater than 100 relative light units (Blue histogram) or greater than the small nonspecific shift seen in cells stained with the secondary antibody only (Red Histogram) were gated further (G2) for detection of TUNEL-FITC staining. (*C*) An overlay of the percentages of $SSEA1^+$ cells stained with TUNEL from one *Δ/Δ* (Blue) and one *Δ*/+ (Red). These 2 histograms demonstrate the major increase in apoptosis in *Δ/Δ* compared to *Δ*/+ PGCs (71.8% vs. 3.8%), and were representative of the average percentages presented by genotype in *D*. (*D*) A graph showing the average percentages of $TUNEL^+/SSEA^+$ cells with standard error bars from the FACS results of 5 to 6 embryos for *Δ/Δ, Δ*/+, and two other possible genotype pooled from 3 separate trials. On average, there was a dramatic difference in the percentage of $TUNEL^+$ PGCs in *Δ/Δ* embryos (71.1%) compared to the 3 other genotypes (3.0-4.1%).

The genotypes of fetuses were determined by PCR retrospectively. While there was no major difference in the small baseline percentage (3-4%) of dying cells between the 3

genotypes possessing at least one functional Oct4 allele, on average over 70% of SSEA-1^+ cells in *Δ/Δ* embryos were TUNEL positive (*D*). However, by gating for only intact cells with high levels of SSEA staining (>2 log shift), our FACS analyses were limited to approximately 10% of the total cell populations and may have excluded additional apoptotic PGCs (*A-C*).

Method: Individual fetuses were isolated from litters of $Oct4^{\Delta/+};TNAP^{Cre/+}$ x $Oct4^{flox/flox}$ mice at 10 days post coitum. The developing head from each fetus was used for DNA extraction and PCR genotyping as above. The ridges of mesenchymal tissue containing migratory PGCs were dissected and digested in DMEM containing 1 mg/ml of Collagenase IV (Sigma) at 37^0 C for 30 minutes. Single-cell suspensions were rinsed with PBS containing 1mg/ml of BSA, screened through a 40μm cell strainer (Falcon) and centrifuged at 1000 RCM. Pellets containing 30-50,000 cells were re-suspended and incubated with the same SSEA-1 antibody (1:200) for 20 minutes on ice and then with a secondary rabbit anti-mouse IgM antibody conjugated with Allophycocyanin-APC (1:200, Jackson Immuno Research). Cell suspensions were fixed in 4% PFA for 20 minutes at room temperature, before performing TUNEL-fluorescein (Fl) staining as above (Roche). Two- color FACS analysis was performed on a dual laser FACScaliber flow cytometer (Becton Dickinson) using Cell Quest™ Software (BD) for data acquisition and FloJo™ (Tree Star, San Carlos, CA) for data analysis.

Suppl. Figure 4. Cre and TUNEL staining in PGCs isolated from individual 10 dpc embryos. (*A, B*) Cell suspensions were stained with TUNEL-fluoroscein to detect fragmented DNA and with an anti-Cre recombinase antibody and a secondary antibody

conjugated with Texas Red. While only TNAP-Cre^{+} PGCs were counted, the genotypes of donor embryos were confirmed by PCR afterwards. Two PGCs from a *Δ/Δ* embryo show Cre staining (Red); the PGC on the right also has an intense, focal TUNEL^{+} spot (Green) that appears yellow in the merged image. (*B, C*) DAPI counter-staining to determine the location of TUNEL staining in apoptotic PGCs. (*B*) The TUNEL (Green) and DAPI (Blue) signals co-localize in the nucleus of the Cre^{+} (Red) *Δ/Δ* PGC at the top; The somatic cell, Cre^{-}/DAPI^{+} (Blue), at the bottom was included to show specificity of the Cre antibody. (*C*) An image of a single Cre+ (Red) *Δ/+* PGC in a cluster of somatic cells, where the TUNEL reaction also labeled the central somatic cell undergoing apoptosis. (*D*) A graph showing the percentage of TUNEL^{+}/Cre^{+} PGCs counted (out of 200) under fluorescent microscopy; the percentages and standard errors of dual-labeled PGCs were calculated from 4 or 5 *Δ/+* and *Δ/Δ* embryos pooled from 3 litters. On average, the *Δ/Δ* embryos had 60% more apoptotic cells than the PGCs with a wild-type Oct4 allele.

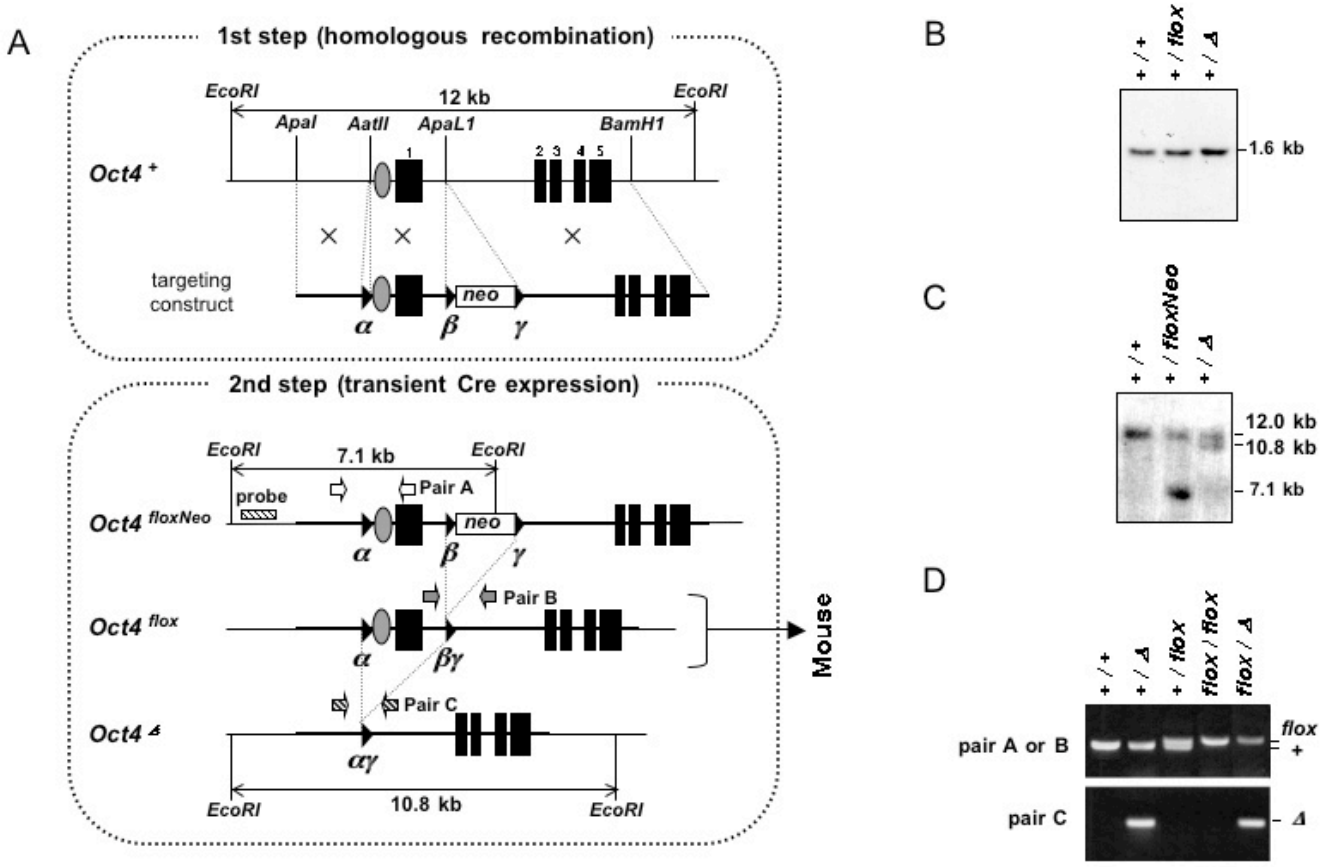
*A one way analysis of variance (ANOVA) performed on actual cell counts showed a significant difference ($p<0.01$) between the mean numbers of apoptotic cells in each genotype. Scale bar in A-C represents 10 μm.

Method: Cells were isolated and prepared as above for FACS analysis (Suppl. Fig. 3). After TUNEL-staining, cells were incubated with a rabbit anti-Cre-recombinase antibody (1:2000, Novagen) in PBS with 5% BSA, 0.1% Tween and 0.2% Triton-X at room temperature for 1 hour, and then with a goat anti-rabbit IgG antibody conjugated with Texas Red at room temperature for 40 minutes (1:500, Jackson Immuno Research). Cell

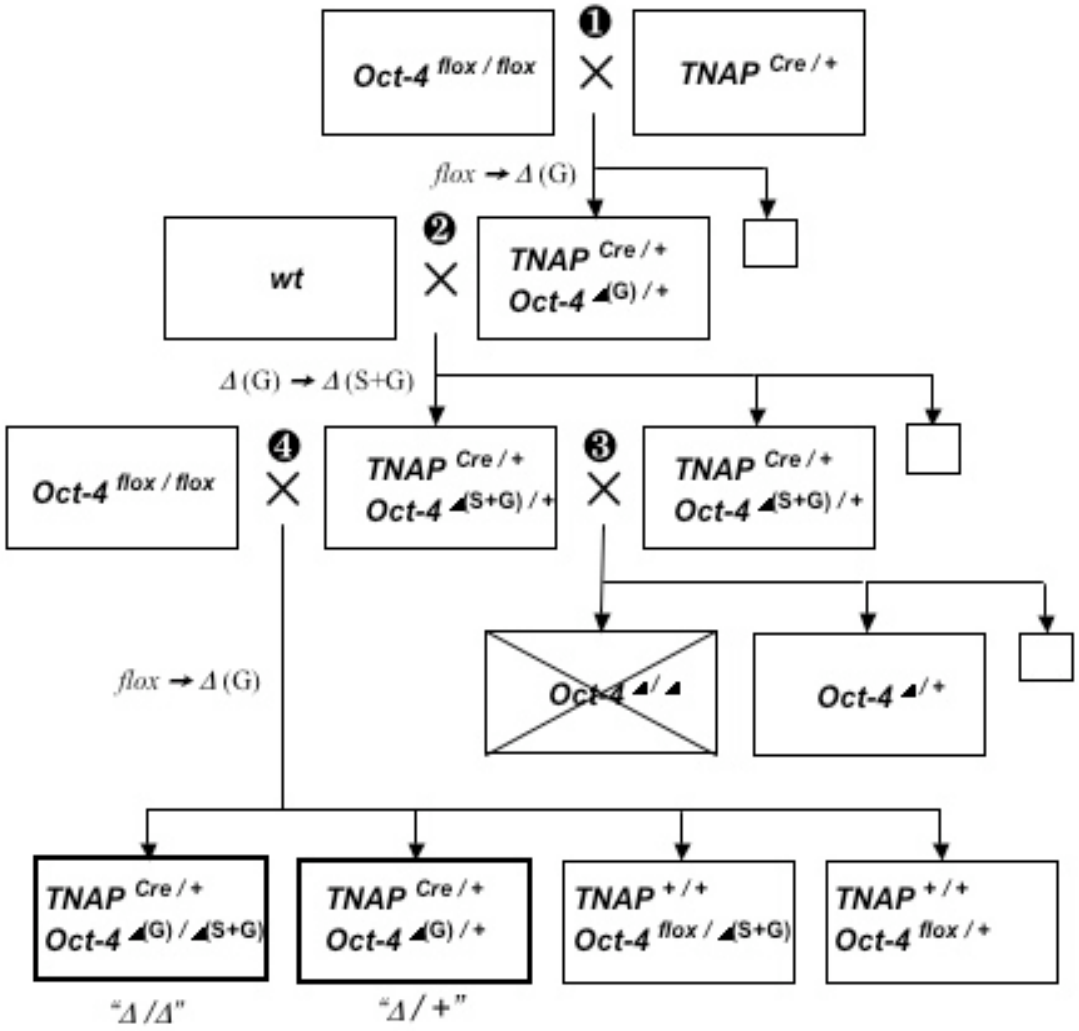
suspensions were stained with 10 ng/ml DAPI (Sigma) for 10 minutes prior to counting on a Leica inverted microscope equipped with epifluorescence. Brightfield and fluorescent images were captured and merged using Openlab™ software (Improvision Coventry, UK). A one-way analysis of variance (ANOVA) was performed on cell counts using JMP™ software (Brooks/Cole Belmont, CA).

Suppliment A

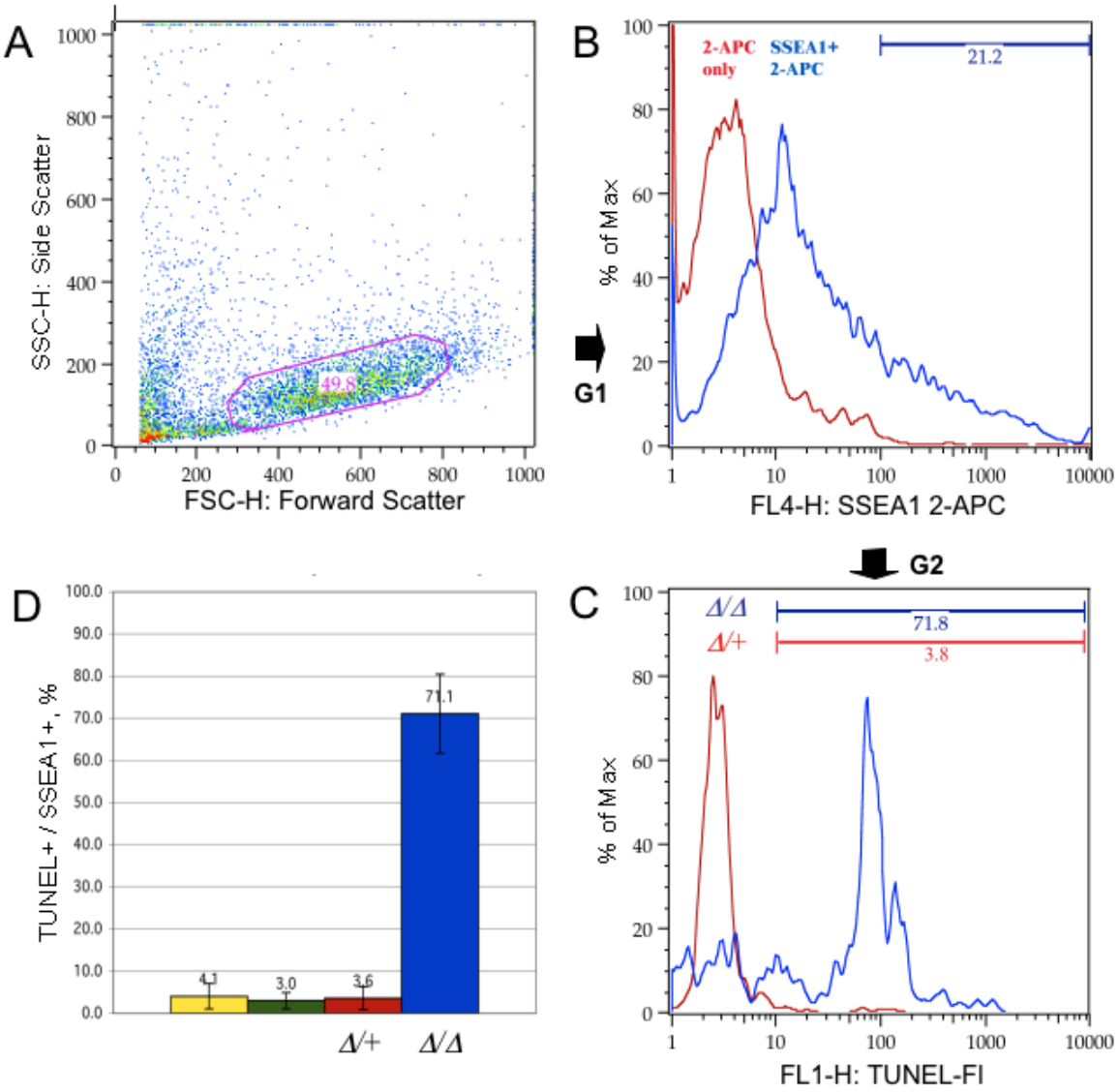
Embryos freed from deciduas and extraembryonic tissues, testes, and ovaries were fixed in 4% paraformaldehyde at room temperature (RT) for 0.5-2 hrs, depending on the specimen size. Testes and ovaries were embedded in paraffin and sectioned (5-7 μm). Embryos were first AP-stained (Ginsburg et al. 1990) and photographed, then processed as described for the adult specimen. Combined fluorescein TUNEL/ SSEA-1 assay was performed using the *In situ* Cell Death Detection Kit (Roche), followed by subsequent 2-hr incubations at RT with SSEA-1 (1:25, Hybridoma Bank) and Alexa594-conjugated goat anti-mouse IgG (H+L) antibodies (1:100, Molecular Probes). Oct-4 staining of a heat-permeabilized paraffin sections was performed using Santa-Cruz monoclonal antibody (1:100), followed by the incubation DakoVision-HRP Mouse reagent (DakoCytomation).



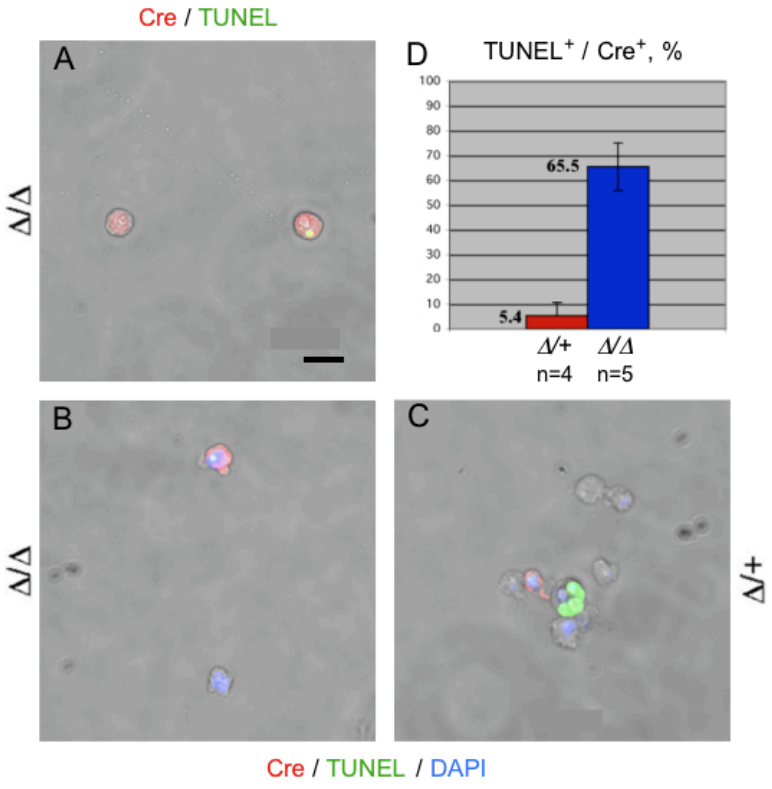
Suppl. Fig. 1 Kehler et al.



Suppl. Fig. 2 Kehler et al.



Suppl. Fig. 3 Kehler et al.



Suppl. Fig. 4 Kehler et al.