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SUPPLEMENTARY MATERIAL

corresponding to:

Dicer is required for Sertoli cell function and survival

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Supplementary Material and Methods

Histologic and immunohistochemical analysis

For histology, embryos and dissected gonads were collected in PBS, fixed in Serra (ethanol:37% formaldehyde:acetic acid, 6:3:1), embedded in paraffin, sectioned to 7 μm, and stained with hematoxylin and eosin. Immunostaining was performed using commercial antibodies from Santa Cruz, CA: OCT-3/4 (sc-8628, 1:100), and DMC1 (sc-8973, 1:100).

Calculation of the total number of PLZF-positive cells per testis

For the approximate calculation of the total number of PLZF-positive cells in wildtype and mutant testes, we prepared consecutive sections of 7 μm thickness, and placed every 20th sections of the wt and mutant testis pairs on a slide. The sections were stained with an anti-PLZF antibody (Santa Cruz, sc-21389, 1:75). The primary antibody was detected using the M.O.M. kit and the ABC kit (both from Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Stained slides were examined with a Zeiss Axioskop 40 microscope. Images were captured by a Zeiss CCD camera. PLZF-positive cells of the largest cross-section of each testis were counted, and the area of the same cross-section was determined using AxioVs40 V 4.6.3.0 (Carl Zeiss Imaging Solutions GmbH). The density of PLZF-positive cells per volume per individual was calculated by dividing the number of positive cells by the volume of the section. Six control and six mutant testes were evaluated per time point. The approximative volume of wildtype and mutant testes for each time point was calculated using the formula:

$$Vol_{testis} = \frac{4}{3}\,\pi abc,$$

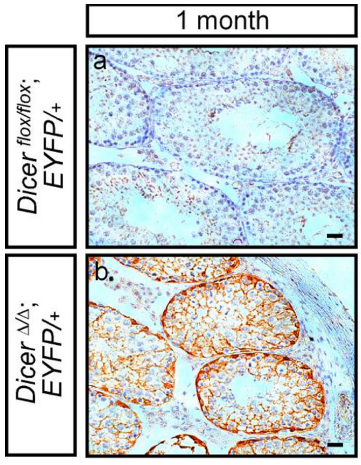
whereby a, b and c represent the radius of the ellipsoid in each axis.

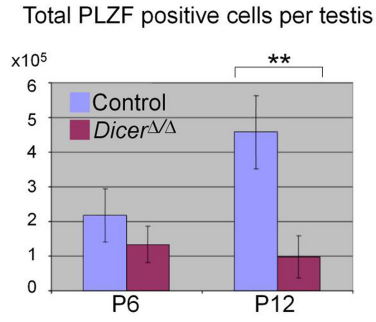
To calculate c, the half-length of the gonad, the number of testis sections on a slide was multiplied by 20 (only each 20th section was placed on slides) and then multiplied by 7 μm (thickness of each section), and finally divided by 2 with an estimated relative error of at least 12% (±2 sections per slide).

The total number of PLZF-positive cells was calculated, using the formula:

$$PLZF_{total} = Vol_{testis} \cdot \frac{PLZF_{counted\_in\_largest\_section}}{Vol_{largest\_section}}.$$

Error bars were calculated according to the Gaussian error calculation (using the s.e.m.s of the averaged values of the three individuals and the estimated error)[1]. *P*-values were determined by performing an unpaired *t*-test using the separately calculated values of the total number of PLZF positive cells for each individual.



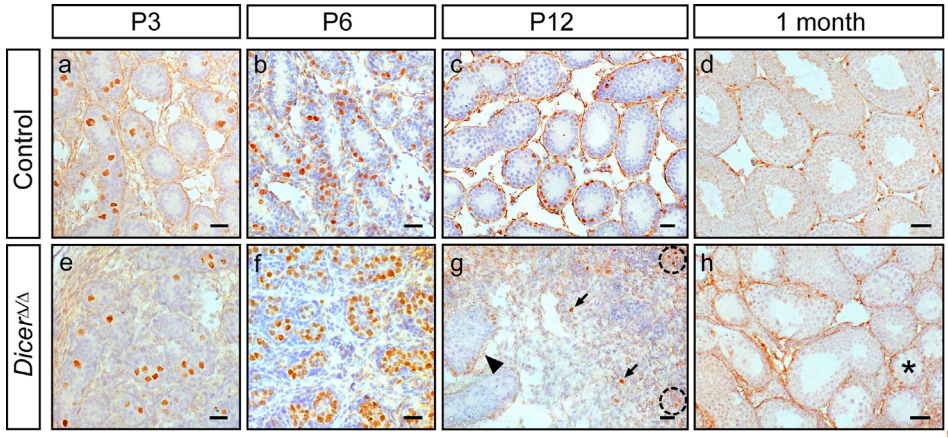


Supplementary Fig. S1. Complete Sertoli cell-specific *AMH-Cre*-mediated recombination in 1 month-old mutant testis. *Recombination of the* R26R-EYFP *Cre reporter allele by the* AMH-Cre *transgene was revealed by IHC for EYFP on 1 month-old* Dicer$^{flox/flox}$;EYFP/+ *control (a) and* Dicer$^{\Delta/\Delta}$;EYFP/+ *mutant testes (b). Note the Sertoli cell-specific cytoplasmic staining in all mutant tubules, revealing a complete and Sertoli cell-specific action of the Cre recombinase. Scale bar, 25 μm.*

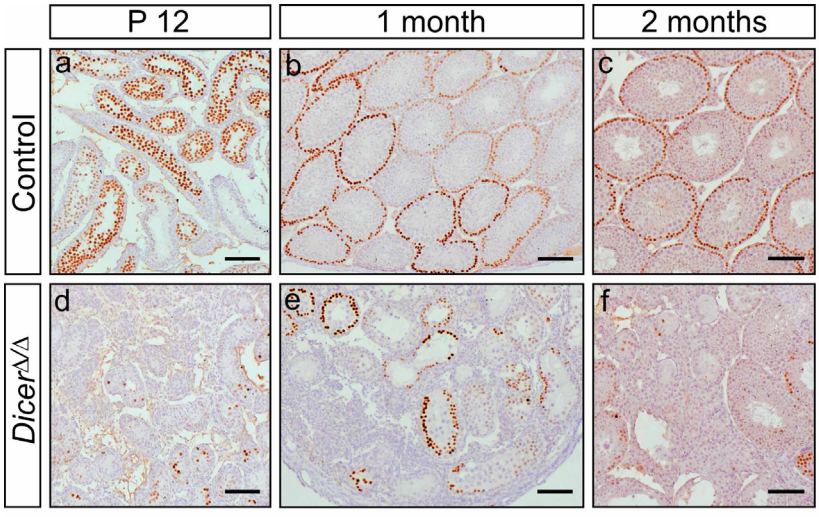
Supplementary Fig. S2. Quantification of PLZF-positive cells per control and *Dicer*$^{\Delta/\Delta}$ testes. *PLZF-positive cells were counted in six control and six mutant testes at P6 and P12, as described under Supplementary Material and Methods above, and used to calculate the total number of such cells per testis. A slight but non-significant decrease at P6 and a significant decrease at P12 in the mutant was observed.*

[1]The formula $\frac{\Delta PLZF_{total}}{PLZF_{total}} = \sqrt{\left(\frac{\Delta Vol_{testis}}{Vol_{testis}}\right)^2 + \left(\frac{\Delta PLZF_{counted\_in\_largest\_section}}{PLZF_{counted\_in\_largest\_section}}\right)^2 + \left(\frac{\Delta Vol_{largest\_section}}{Vol_{largest\_section}}\right)^2}$ was used.

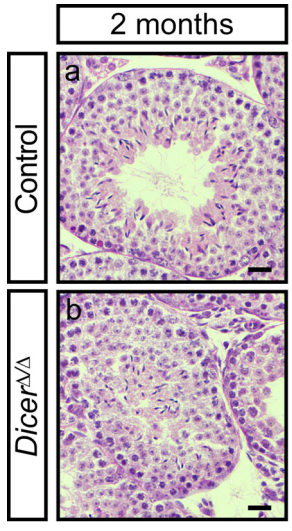
For the calculation of the relative errors, the s.e.ms of the averaged values were used. For the calculation of the half length of the gonad, a relative error of at least ~12% was assumed (±2 sections per slide).



Supplementary Fig. S3. Progressive loss of spermatogonial stem cells in *Dicer*$^{\Delta/\Delta}$ testes revealed by IHC for OCT4. *Control (a-d) and* Dicer$^{\Delta/\Delta}$ *testes (e-h) stained by immunohistochemistry (IHC) for the SSC marker OCT4. (a,e) At P3, OCT4 expression was similar in control and mutant testis cords. (b-d) OCT4-positive cells were located at the periphery of control tubules at P6, P12 and 1 month. (f) P6 mutant testis tubules displayed a wildtype pattern of OCT4-positive cells. (g) At P12, mutant testes contained tubules with a normal pattern of expression (arrowhead), clusters of stained cells surrounded by peritubular myoid cells (dashed circles), and single positive cells outside of the tubules (arrows). (h) At 1 month, of the few remaining tubules, only very few contained OCT4-positive cells (asterisk). Scale bar, 25 μm (a-c, e-g); 50 μm (d,h).*



Supplementary Fig. S4. Spermatogenesis is delayed and defective in *Dicer*$^{\Delta/\Delta}$ testes as revealed by IHC for DMC1. *Control (a-c) and* Dicer$^{\Delta/\Delta}$ *testes (d-f) stained by immunohistochemistry (IHC) for the meiosis-specific DMC1 DNA strand exchange protein that marks leptotene-to-zygotene spermatocytes. (a) Synchronous onset of spermatogenesis at P12 as indicated by DMC1-expressing spermatocytes filling the tubules in the control. (d) A low number of DMC1-positive spermatocytes mark an asynchronous and delayed onset of spermatogenesis in the P12* Dicer-*mutant. (b,c) One and two month-old control testes show ongoing spermatogenesis and display DMC1-positive spermatocytes exclusively at the tubule periphery. (e) In 1 month-old mutant testes, only some tubules show DMC1-positive spermatocytes at their periphery, some tubules show a disordered DMC1-positive spermatocyte distribution, while most tubules are DMC1-negative. (f) Mutant tubules at 2 months are mostly DMC1-negative, revealing a progressive defect in spermatogenesis. Scale bar, 100 μm.*



Supplementary Fig. S5. Rare *Dicer*$^{\Delta/\Delta}$ tubule at 2 months with elongated and elongating spermatids. *(a) Control tubule with elongated and elongating spermatids and central lumen. (b) Mutant tubule lacking a central lumen with elongated and elongating spermatids. HE staining. Scale bar, 20 μm.*