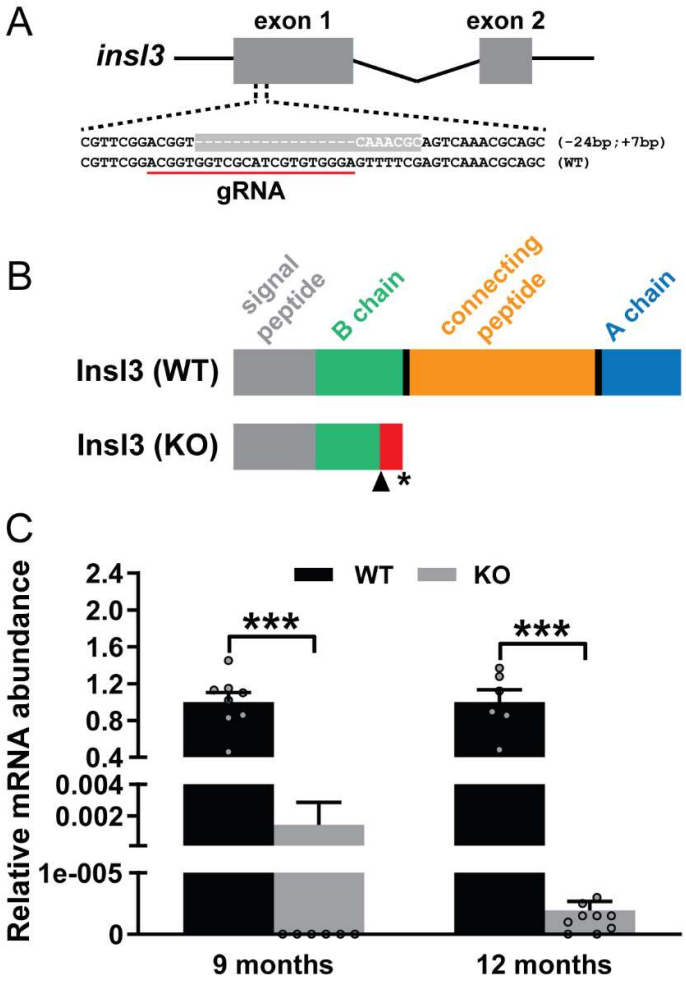
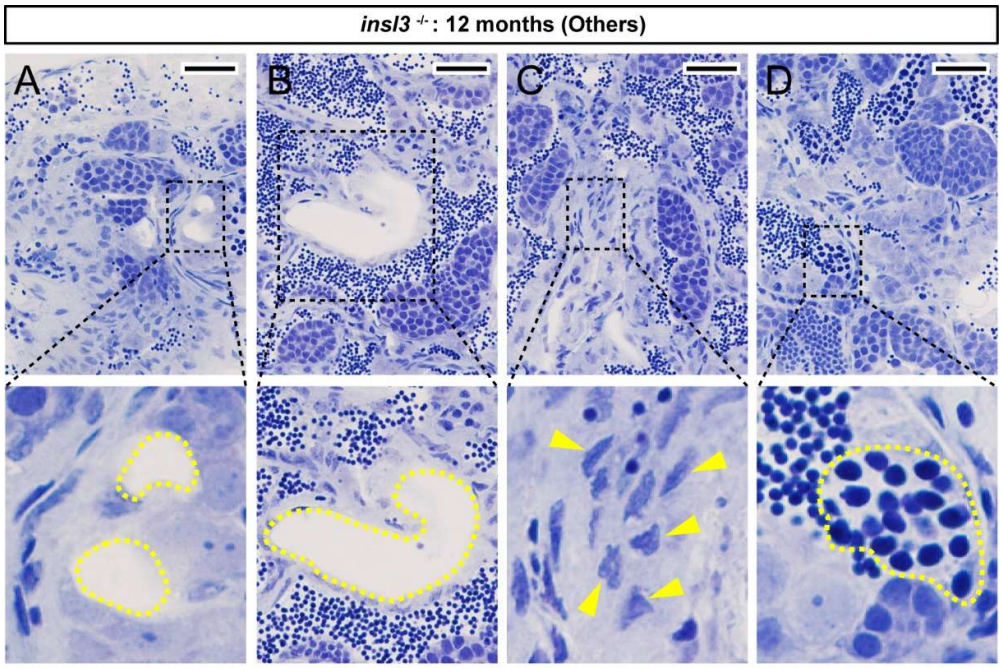
Supplementary Information

Insulin-like 3 affects zebrafish spermatogenic cells directly and via Sertoli cells

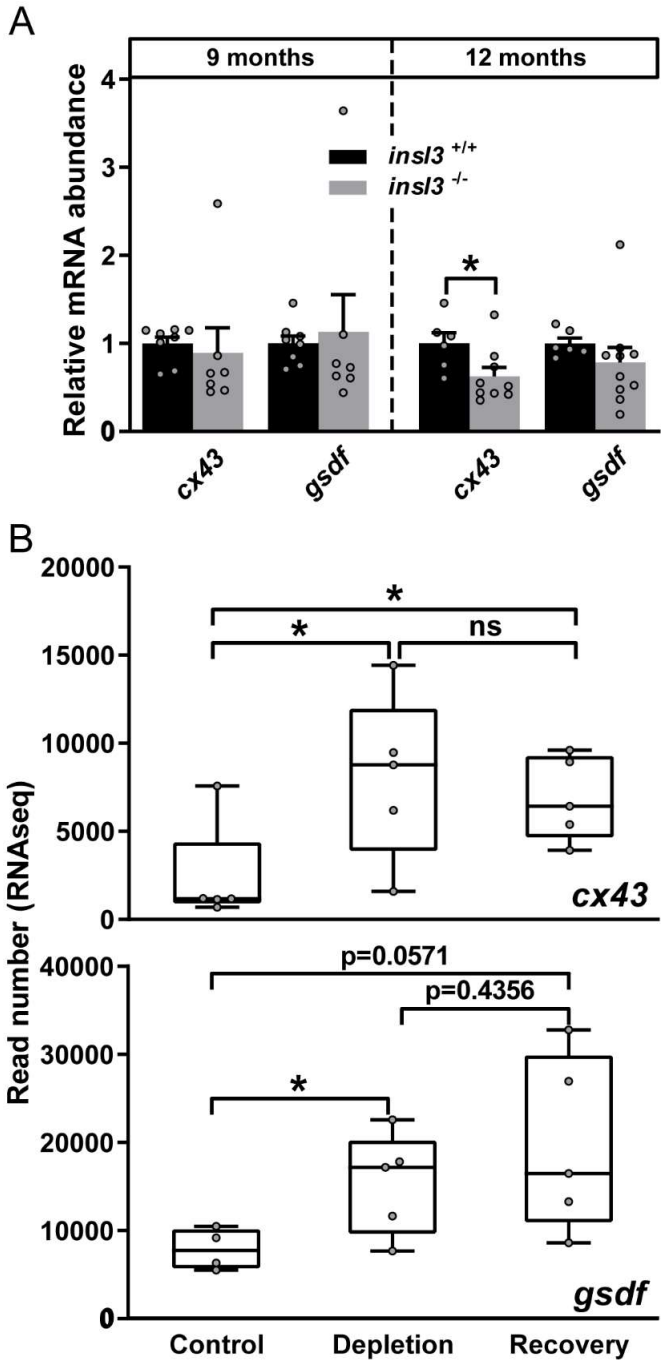
Diego Crespo, Luiz H. C. Assis, Yu Ting Zhang, Diego Safian, Tomasz Furmanek, Kai Ove Skaftnesmo, Birgitta Norberg, Wei Ge, Yung-Ching Choi, Marjo J. den Broeder, Juliette Legler, Jan Bogerd, Rüdiger W. Schulz



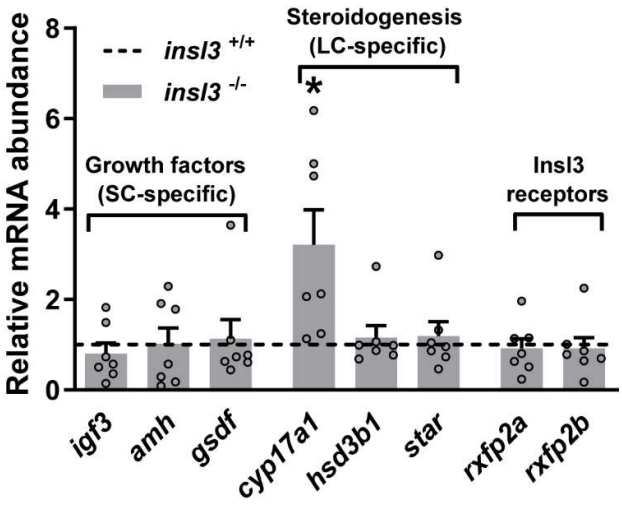
Supplementary Fig. 1 Efficient disruption of zebrafish *insl3* by CRISPR/Cas9. a Design of the guide RNA (gRNA) target site on exon 1 targeting the *insl3* gene (underlined sequence). Representative Sanger sequencing results showing the indels identified (highlighted with a grey background) in the *insl3* mutant line generated in this study. Numbers to the right of the mutant sequence indicate the loss or gain of bases, with the number of bases deleted (-) or inserted (+). WT, wild-type; bp, base pair. **b** Schematic diagram showing the functional domains of the WT Insl3 protein *versus* the *insl3* knockout (KO) mutant sequence. The black arrowhead indicates the start of the indel (the insertion indicated with the red box) and the asterisk indicates a premature stop codon caused by the frame shift. Black boxes in the Insl3 precursor indicate proteolytic processing sites. **c** CRISPR/Cas9-mediated decrease of *insl3* transcript levels in both 9 and 12 month-old zebrafish KO testes. Data are shown as mean fold change ± SEM (WT and KO, 9 months: N = 8 and 7; WT and KO, 12 months: N = 6 and 10; ***, $p < 0.001$) and expressed relative to the WT control group (which is set at 1).



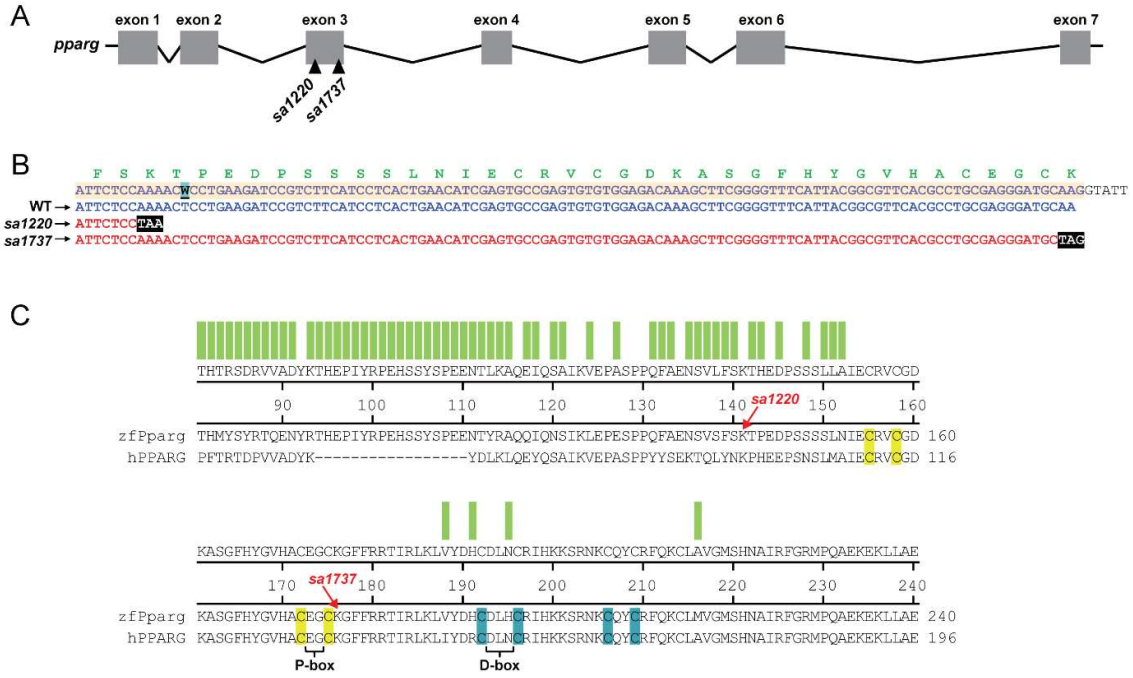
Supplementary Fig. 2 Composition of the category "Others" shown in Fig. 2d. a-d Areas found exclusively (**a**-**c**) or more prominently (**d**) in 12 months-old *insl3* knockout (*insl3*$^{-/-}$) testes. **a** Empty areas adjacent to Sertoli and/or germ cells within the germinal epithelium. **b** Empty areas lined by cytoplasmic extensions of Sertoli cells within the germinal epithelium. **c** Areas containing an accumulation of Sertoli cells. **d** Areas containing apoptotic germ cells. The stippled black areas identify the magnified areas. The stippled yellow lines in **a** indicate empty space lined by germ and/or Sertoli cells, or lined only by Sertoli cell extensions in **b**, in both cases not forming part of the tubular lumen. Yellow arrowheads in **c** indicate areas showing an accumulation of only Sertoli cells. The yellow stippled line in **d** indicates a group of germ cells (type B spermatogonia) that show the typical appearance of apoptotic cells in toluidine-blue stained plastic sections (shrinkage, losing at least partially the contact with the immediate tissue environment, pyknosis/nuclear fragmentation[3]). Scale bars, 25 µm.



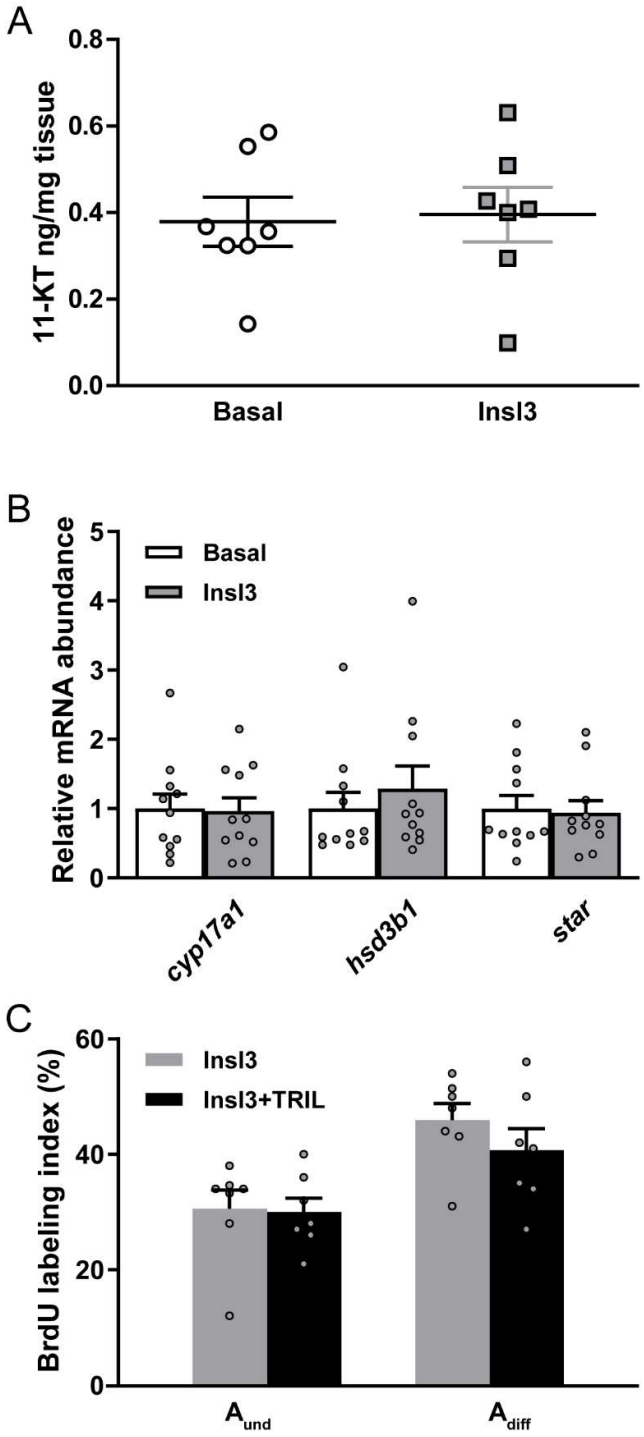
Supplementary Fig. 3 Testicular expression of selected Sertoli cell genes in *insl3* knockout mutant gonads, and in control, depleted and recovering gonads. a *cx43* and *gsdf* transcript levels in wild-type (*insl3*$^{+/+}$) and *insl3* knockout (*insl3*$^{-/-}$) testes 9 and 12 months post-fertilization. Data are shown as mean fold change ± SEM (*insl3*$^{+/+}$ and *insl3*$^{-/-}$, 9 months: N = 8 and 7; *insl3*$^{+/+}$ and *insl3*$^{-/-}$, 12 months: N = 6 and 10; *, $p < 0.05$) and expressed relative to the wild-type control group (which is set at 1). **b** Expression levels of *cx43* and *gsdf* in control, germ cell-depleted (by exposure to the cytostatic agent busulfan[1]), and testes with recovering (from busulfan) spermatogenesis, as described by Crespo et al.[2] (NCBI GEO data set GSE116611). Data are expressed as mean ± SEM (N = 5; *, $p < 0.05$). ns, not significant differences between groups.



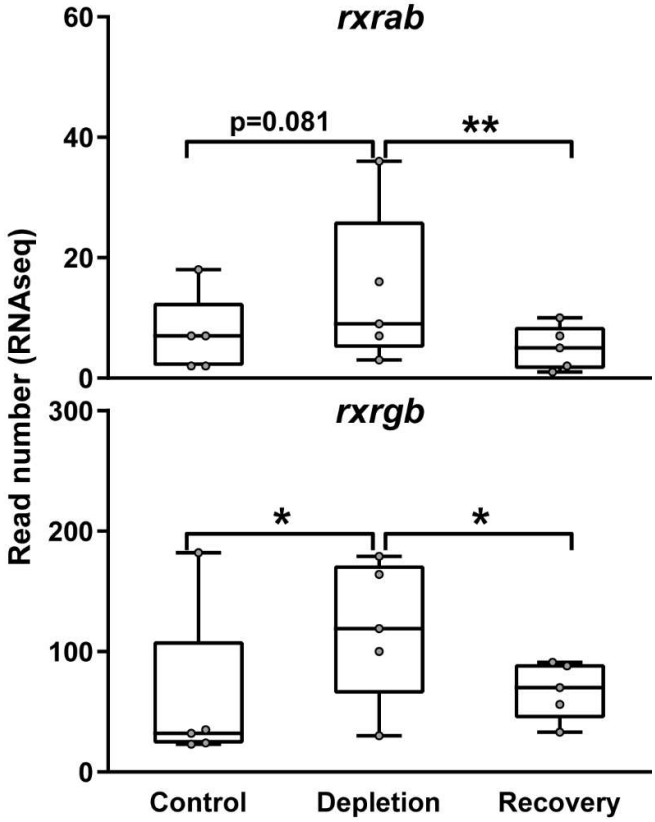
Supplementary Fig. 4 Testicular expression of selected genes in 9 month-old *insl3* CRISPR-ed zebrafish. Transcript levels of growth factors, steroidogenesis-related and Insl3 receptors in 9 month-old wild-type (*insl3*$^{+/+}$) and *insl3* knockouts (*insl3*$^{-/-}$) adult testis tissue. Data are mean fold change ± SEM (*insl3*$^{+/+}$ and *insl3*$^{-/-}$, N = 8 and 7; * $p < 0.05$) and expressed as relative to the wild-type group (which is set at 1; dashed line). SC, Sertoli cell; LC, Leydig cell.



Supplementary Fig. 5 Schematic description of the zebrafish mutant lines for *pparg* obtained from the Zebrafish International Resource Center (ZIRC). a Diagram showing both point mutations (*sa1220* and *sa1737*) in exon 3 of the *pparg* gene, as indicated by the black arrowheads. **b** Alignment showing the wild-type (WT) *pparg* nucleotide sequence *versus* the *pparg* knockout mutant sequences. Both mutant *pparg* alleles have an A>T nonsense mutation leading to a premature stop codon (black background) at amino acid 141 (*pparg*$^{-/-\ sa1220}$) or amino acid 176 (*pparg*$^{-/-\ sa1737}$). **c** Amino acid alignment of human and zebrafish PPARG/Pparg proteins, showing amino acid disagreement (green bars), the cysteine residues constituting zinc finger 1 (yellow) and zinc finger 2 (cyan). The Pparg protein in mutant *sa1220* lacks both zinc fingers of the DNA-binding domain as well as the ligand-binding domain (LBD), while the Pparg protein in mutant *sa1737* retains the first zinc finger, including the P-box (responsible for sequence-specific DNA recognition), but lacks the second zinc finger, which harbours the D-box (involved in protein-protein cooperative interaction in the dimerization process) and the subsequent LBD.



Supplementary Fig. 6 Insl3-induced effects on spermatogenesis are steroid-independent in zebrafish. a-b 11-ketotestosterone (11-KT) levels in medium (**a**) and steroid-related gene expression in testis tissue (**b**) after incubation in the absence or presence of 100 ng/mL Insl3 for 4 days. **c** Evaluation of the proliferation activity of type A spermatogonia in zebrafish testes cultured for 4 days with 100 ng/mL Insl3, and in the absence or presence of trilostane (TRIL; 25 μg/mL). In **a** and **c**, data are shown as mean ± SEM (N = 7), and in **b** as mean fold change ± SEM (N = 11) and expressed relative to the basal control group (which is set at 1).



Supplementary Fig. 7 Testicular expression of selected retinoid X receptors in control, depleted and recovering gonads. Expression levels of *rxrab* and *rxrgb* in control, germ cell-depleted (by exposure to the cytostatic agent busulfan[1]), and testes with recovering (from busulfan) spermatogenesis, as described by Crespo et al.[2] (NCBI GEO data set GSE116611). Data are expressed as mean ± SEM (N = 5; *, $p < 0.05$; *, $p < 0.01$).

Supplementary Table 1 Sequences of primers used for the generation of transgenic lines.

Transgenic line	Primers	Primer sequence (5′→ 3')
Tg(rxfp2a:EGFP): zebrafish *rxfp2a* promoter (3193 bp, preceding the ATG start codon)	4703 (Fw)	TAGGCCGGCCTATCACTGAGCTTTATCTAAAGATTTTTATAAT
	4704 (Rv)	TTCGGCGCGCCAGTGAAGGCTTTACAAAAGCATGACCGA
Tg(rxfp2b:mCherry): zebrafish *rxfp2b* promoter (3087 bp, preceding the ATG start codon)	4705 (Fw)	TAGGCCGGCCTCATAATTAAACCTACCGTTATGAAACTGAAGTACC
	4706 (Rv)	TTCGGCGCGCCTGTGGCGTCGTGCCAGTCATGC

Fse I restriction enzyme site; Asc I restriction enzyme site.

Supplementary Table 2 Sequences of primers used in gene expression analyses by qPCR.

Target gene	Gene description	Primers	Primer sequence (5′→ 3')
aldh1a2	aldehyde dehydrogenase 1 family, member A2	4359 (Fw) 4360 (Rv)	CGCTGGATGGGCAGATAAGA TCTGGTGAGGGTGAAAAATTCTC
amh	anti-Mullerian hormone	4486 (Fw) 4487 (Rv)	CTCTGACCTTGATGAGCCTCATTT GGATGTCCCTTAAGAACTTTTGCA
casp9	caspase 9, apoptosis-related cysteine peptidase	6169 (Fw) 6170 (Rv)	ACATACGACTGCTGTGTGGTCAT GAACTGCACCAGGGAATCGA
cyp17a1	cytochrome P450, family 17, subfamily A, polypeptide 1	2773 (Fw) 2774 (Rv)	GGGAGGCCACGGACTGTTA CCATGTGGAACTGTAGTCAGCAA
cyp26a1	cytochrome P450, family 26, subfamily A, polypeptide 1	4383 (Fw) 4384 (Rv)	TGGGCTTGCCGTTCATTG CATGCGCAGAAACTTCCTTCTC
eef1a1l1	eukaryotic translation elongation factor 1 alpha 1, like 1	2476 (Fw) 2477 (Rv)	GCCGTCCCACCGACAAG CCACACGACCCACAGGTACAG
gsdf	gonadal somatic cell derived factor	2366 (Fw) 2367 (Rv)	CATCTGCGGGAGTCATTGAAA CAGAGTCCTCCGGCAAGCT
hsd3b1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	5096 (Fw) 5097 (Rv)	GATCCGACTGCTGGATAGAAACA CCCGGCAATCATCAAGAGA
insl3	insulin-like 3 (Leydig cell)	2466 (Fw) 2467 (Rv)	TCGCATCGTGTGGGAGTTT TGCACAACGAGGTCTCTATCCA
rpl13a	ribosomal protein L13a	3998 (Fw) 3999 (Rv)	GAGCCCCCAGCAGAATCTTC AGCCTGACCCCTCTTGGTTTT
rxfp2a	relaxin family peptide receptor 2a	3258 (Fw) 3259 (Rv)	CAATTCCAGTCTCTGTCAGCACAT CTCAACGTCATTCTCCGCAAA
rxfp2b	relaxin family peptide receptor 2b	3262 (Fw) 3263 (Rv)	CTGCCAGACTCTGTGCCCATA AGTCGTGATGCTATTACCCTCGAA
star	steroidogenic acute regulatory protein	2546 (Fw) 2547 (Rv)	CCTGGAATGCCTGAGCAGAA ATCTGCACTTGGTCGCATGAC
ubc	ubiquitin C	3951 (Fw) 3952 (Rv)	CCATACACCGCACTCTTACAGAAA CCAGTCAGCGTCTTCACAAAGAT
xiap	X-linked inhibitor of apoptosis	6159 (Fw) 6160 (Rv)	CATCCCATTGACCCTGAGAGA CCTCCACATCTGAAGCACATCA

Fw, forward; Rv, reverse.

References

1 Nobrega, R. H. *et al.* Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS One* **5**, doi:10.1371/journal.pone.0012808 (2010).

2 Crespo, D. *et al.* Endocrine and local signaling interact to regulate spermatogenesis in zebrafish: follicle-stimulating hormone, retinoic acid and androgens. *Development* **146**, doi:10.1242/dev.178665 (2019).

3 Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516, doi:10.1080/01926230701320337 (2007).