Supporting Information

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SI Materials and Methods

Fish. Adult Senegalese sole, gilthead seabream, and zebrafish were obtained from aquaculture research centers in Spain and local pet stores and maintained following standard husbandry practices. All experiments with live animals were performed using protocols approved by the European Union Council Guidelines (86/609/EU) and the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) Ethics Committee.

Preparation of Testis and SLL Cell Extracts. Testes samples (~10 mg) were cut into small pieces and treated with 0.2% collagenase (type 1A; Sigma) for 1 h under agitation in Leibovitz's L-15 culture medium without phenol red (Life Technologies Corp.) and supplemented with 10 mM Hepes (Sigma), 0.5% BSA (Fraction V, Sigma), 0.4 mg/mL Fungizone (Sigma), and 200 μg/mL penicillin/streptomycin (Life Technologies Corp.). Samples were centrifuged at 200 × *g*, and the supernatant was diluted 1:100 in L-15 before flow cytometry or FACS.

To isolate the cells of the seminiferous lobule lumen (SLL), whole testes (0.6–2.5 mg) were excised and washed in PBS + 1 mM $MgCl_2$ + 1 mM $CaCl_2$ and transferred to a Petri dish containing Leibovitz's L-15 culture medium. The testis pieces were then gently stripped with forceps in an 1.5-mL Eppendorf tube containing 300 μL L-15 and centrifuged at 200 × *g* to recover only free cells within the SLL. Cell concentration was evaluated by light microscopy and the ISASv1 software (Proiser).

Flow Cytometry. Quantitative flow cytometry was performed on testicular and SLL cell suspensions with a Becton-Dickinson FACSCalibur machine with a blue-light (488 nm) laser operating at 15 mW and a bandpass filter at 530/30 nm (green fluorescence). Approximately 200 μL of the diluted testis and SLL samples were stained with 200 nM of a solution of SYBR Green I (SGI) fluorescent nucleic acid stain (Molecular Probes, Life Technologies Corp.) for 10–15 min in the dark at room temperature. The samples were run at low speed (*ca*. 15 mL/min) with MilliQ water as sheath fluid. Enumerations of around 10,000 living cells per sample were carried out on the basis of their DNA content. Cells were detected in a plot of size scatter (SSC) versus green fluorescence. The threshold was applied to green fluorescence, and acquisition was done in logarithmic mode. The DNA histograms were analyzed with the CellQuest (Becton-Dickinson) software.

FACS was performed with a Becton-Dickinson FACSAria II flow cytometer using a 100-mW laser at 488 nm with bandpass filters at 525/50 nm. The instrument was prepared for sorting following the Aseptic Sort procedure (1). Sterilized PBS served as the sheath fluid. The sorter was set in 4-way purify sort mode and with a flow sorting rate of 467 events/s. The sorted populations (~100,000 cells per population) were collected in L-15 medium.

Culture of Senegalese Sole SLL Extracts and Spermatids in Vitro. SLL cell extracts and FACS-purified spermatids diluted in L-15 (~2×10^6 cells/mL and ~2×10^5 cells/mL, respectively) were loaded in 48-well tissue plates and incubated in triplicate with recombinant follicle-stimulating hormone (rFsh) or recombinant luteinizing hormone (rLh) produced as previously described (2), forskolin (FSK), or steroids (Sigma), with or without different inhibitors (Sigma, Cayman Chemical Company, or Selleck Chemicals), at 18 °C up to 72 h. The Fshra and the homolog of the tetrapod luteinizing hormone/choriogonadotropin receptor (Lhcgrba) antibodies were used in 0.5% DMSO. Controls were exposed to the vehicle solutions at the same concentration. At different times, 5-μL aliquots of the samples were analyzed by quantitative flow cytometry.

Gene Expression Analyses. RT-PCR and quantitative RT-PCR were carried out as previously described (3). Relative gene expression levels with respect to those at time 0 were determined by the $2^{-\Delta\Delta Ct}$ method, using elongation factor 1 (*efa1*) as a reference gene. Primer sequences are listed in Table S1. In situ hybridization was carried out as described previously (2). Zebrafish and gilthead seabream *lhcgrba* probes were synthesized from GenBank accession numbers NM_205625 and AY587261, respectively.

Histology, Immunofluorescence, and Immunoblotting. Testis samples from males injected intramuscularly with His-tagged rLh (6 μg/kg) or vehicle, as well as from untreated males, were processed as described previously (2). The SLL crude extracts and FACS-sorted cells were attached to UltraStick/UltraFrost Adhesion slides (Electron Microscopy Sciences) and fixed in 4% paraformaldehyde (PFA) for 15 min. Zebrafish and seabream testis sections were subjected to antigen retrieval before blocking by using cold acetone (−20 °C) for 10 min, followed by two washes in PBS and three consecutive 5-min incubations with boiling citrate (10 mM at pH 6). The antibodies were Senegalese sole Lhcgrba (1:500), α-tubulin (Sigma T9026; 1:1,000), H3K9ac (Abcam ab4441; 1:1,000), and H4K12ac (Upstate 07–595; 1:1,000); piscine Lhβ subunit [(4); 1:500)]; and 6xHis tag (Clontech 631212; 1:500). Sections were counterstained with DAPI. Immunoblots for Lhcgrba and Fshra were carried out as previously described (2).

Statistics. Results are expressed as the means ± SEM. Data were analyzed by one- or two-way ANOVA, after log-transformation of the data when needed, followed by the Duncan's multiple range test. All experiments were repeated at least three times using different fish. A value of $P < 0.05$ was considered statistically significant.

1. McIntyre CA, Flyg BT, Fong TC (2010) Fluorescence-activated cell sorting for CGMP processing of therapeutic cells. *BioProcess Int* 8:44–53.
2. Chauvigné F, et al. (2012) Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionary advanced teleost. *Biol Reprod* 87(2):35.
3. Chauvigné F, et al. (2010) Functional and evolutionary analysis of flatfish gonadotropin receptors reveals cladal- and lineage-level divergence of the teleost glycoprotein receptor family. *Biol Reprod* 82(6):1088–1102.
4. Mateos J, Mañanós E, Swanson P, Carrillo M, Zanuy S (2006) Purification of luteinizing hormone (LH) in the sea bass (Dicentrarchus labrax) and development of a specific immunoassay. *Cienc Mar* 32:271–283.

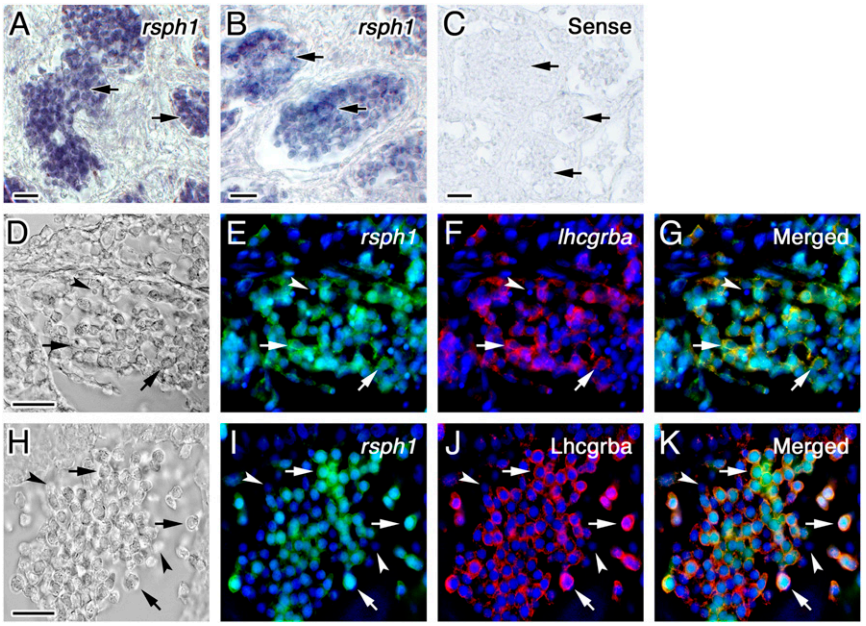

Fig. S1. Senegalese sole Lhcgrba mRNA and protein product colocalizes with the spermatid-specific marker radial spoke head protein 1 (*rsph1*). (*A*–*C*) Localization of *rsph1* expression in spermatids (arrows) in the cortex (*A*) and medulla (*B*) regions of the sole testis by in situ hybridization. Sense probes (*C*) were negative. Bright-field (*D* and *H*) and epifluorescence (*E*, *F*, *I*, and *J*) photomicrographs showing double in situ hybridization for *rsph1* (*E*) and *lhcgrba* (*F*) or combined *rsph1* in situ hybridization (*I*) and Lhcgrba immunofluorescence (*J*) on released spermatids. *G* and *K* show the merge of the cyanine 3 (Cy3; green color) and tetramethylrhodamine (TRITC; red color) channels for the indicated samples. In all panels, cell nuclei were stained with DAPI (blue). Arrows indicate spermatids whereas arrowheads indicate spermatozoa. (Scale bar, 15 μm.)

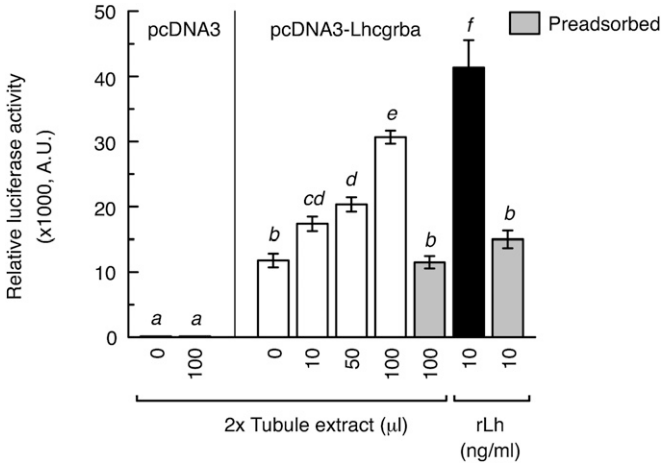

Fig. S2. Effect of the SLL fluid on HEK293T cells transiently transfected with empty plasmid or containing the sole Lhcgrba, together with pCRE-luc. As a positive control, cells were treated with rLh. The highest doses of the SLL fluid and rLh were preincubated with a piscine (*Dicentrarchus labrax*) Lhβ antiserum before addition to the cells. Data (mean ± SEM; $n = 3$ independent transfections) with different superscript are significantly different ($P < 0.05$).

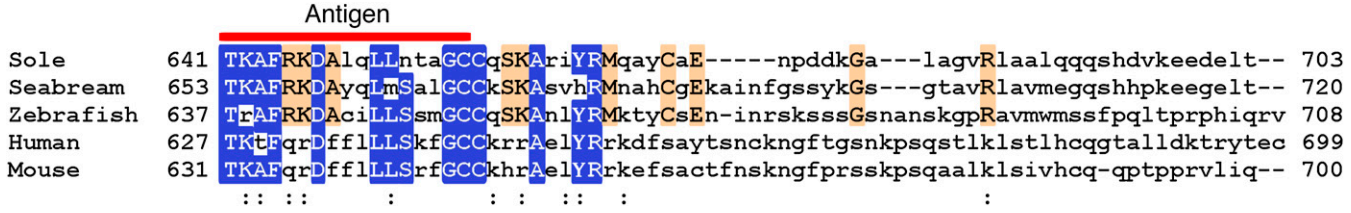

Fig. S3. C-terminus amino acid sequence alignment of teleost (Senegalese sole, gilthead seabream, and zebrafish) and tetrapod (human and mouse) luteinizing hormone/choriogonadotropin receptor. The red line above the sole sequence indicates the antigenic peptide used for antibody production. Amino acids highlighted in blue are conserved across the species, whereas those highlighted in orange are conserved only in teleosts.

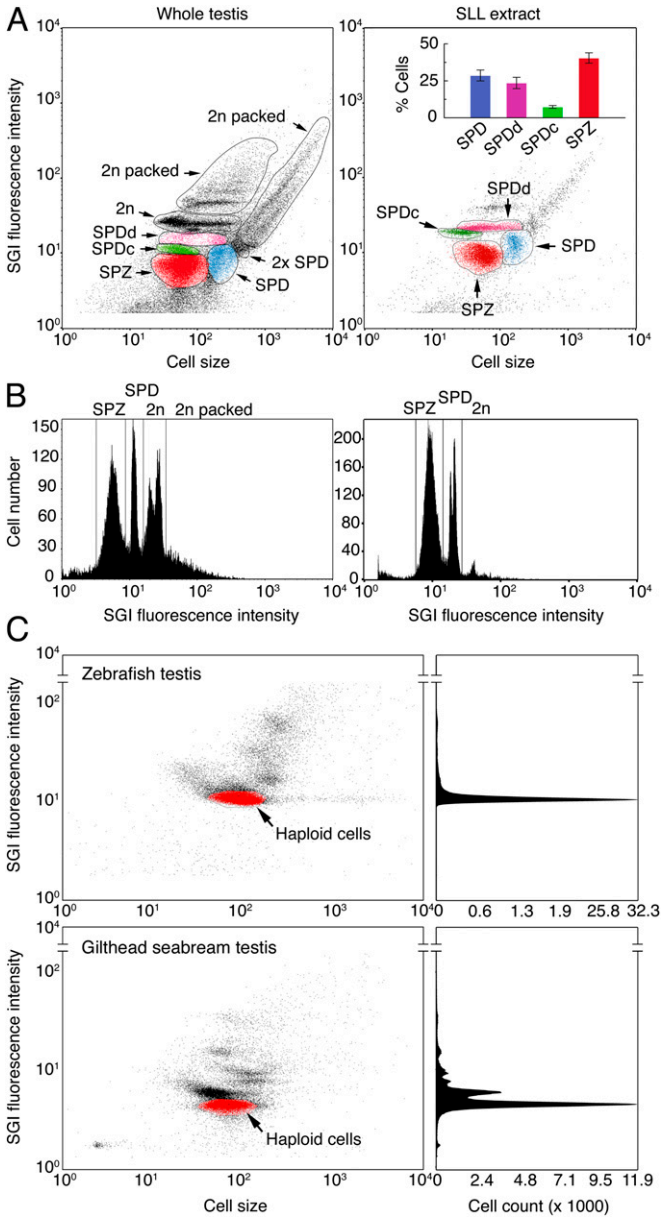

Fig. S4. Flow cytometry of Senegalese sole, zebrafish, and gilthead seabream testes. (*A*) Representative flow cytometry plots of the sole cell populations in the whole testis (left) or in the SLL (right) cell extracts indicating four major populations of haploid cells in the SLL corresponding to different stages of spermatid differentiation into spermatozoa. Cells are separated by SGI fluorescence intensity and side scatter, a surrogate of cell size. The inset shows the percentage (mean ± SEM; $n = 12$ fish) of the different haploid cells recovered from the SLL. (*B*) Flow cytometry histograms of SGI fluorescence of the same samples as in *A*. (*C*) Representative flow cytometry plots (*Left*) and SGI histograms (*Right*) of the different cell populations identified in extracts from the zebrafish and gilthead seabream testis. Cells are separated by SGI fluorescence intensity and side scatter (*Left*) or by SGI fluorescence intensity (*Right*). Sorted haploid cells are highlighted in red. SPD, spermatids; SPDd, differentiating spermatids; SPDc, condensed spermatids; SPZ, spermatozoa; 2n, diploid cells.

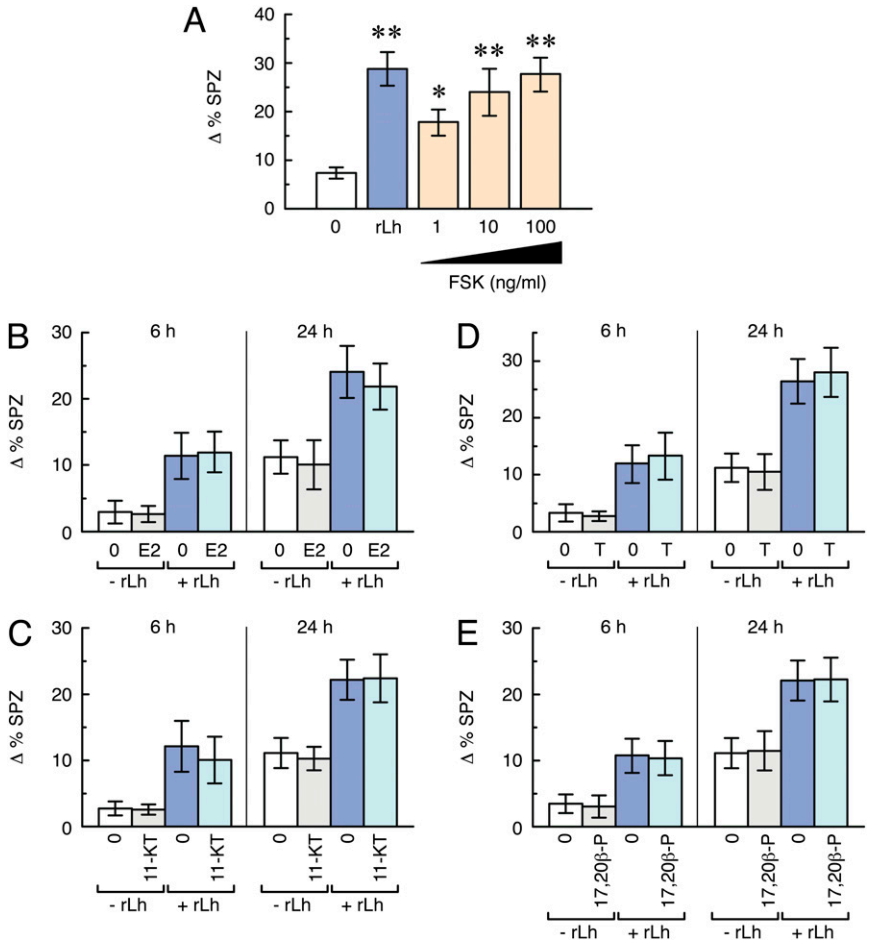

Fig. S5. Effect of FSK and steroid hormones, estradiol-17β (E2), testosterone (T), 11-ketostestoterone (11-KT), and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), on the differentiation of SLL spermatids to spermatozoa in vitro determined by quantitative flow cytometry. (*A*) Percentage of differentiated spermatozoa after 24 h of culture in the presence of rLh (100 ng/mL) or increasing doses of FSK (0–100 ng/mL). (*B–E*) Effect of 10 ng/mL E2, T, 11-KT, or 17,20β-,P in the presence (+) or absence (–) of 10 ng/mL rLh, on differentiated spermatozoa. Data (mean ± SEM; $n = 4$) are the increment in the percentage of spermatozoa with respect to time 0. *$P < 0.05$; **$P < 0.001$, with respect to untreated cells. SPZ, spermatozoa.

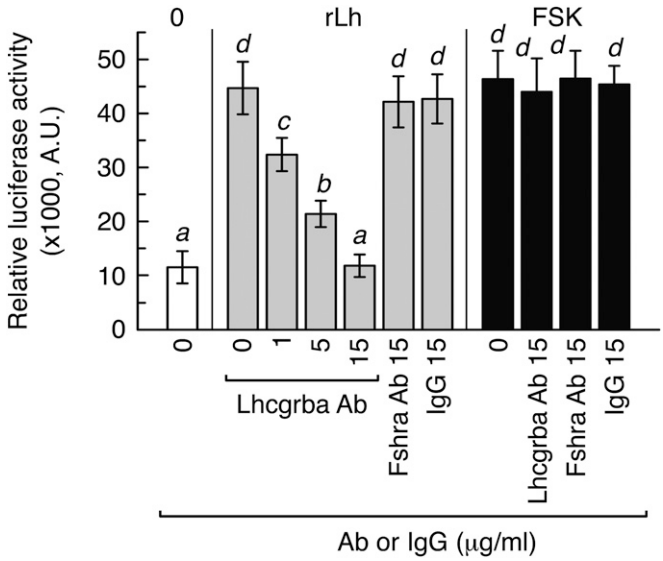

Fig. S6. Activation of sole Lhcgrba expressed in HEK293T cells by rLh is blocked specifically by the Lhcgrba antibody. Cells were transiently transfected with the pcDNA3-Lhcgrba and β-galactosidase (β-Gal) plasmids, together with the cAMP-responsive reporter gene vector (pCRE-luc), and exposed to the vehicle or 10 ng/mL rLh or FSK. Some cells were preincubated for 1 h with the Lhcgrba (Lhcgrba Ab; 1–15 μg/mL) or Fshra (Fshra Ab; 15 μg/mL) antibodies or IgG (15 μg/mL) before treatment with rLh or FSK. Luciferase activity was normalized to β-Gal activity. Data (mean ± SEM; $n = 3$ independent transfections) with different superscript are significantly different ($P < 0.05$).

Table S1. RT-PCR and quantitative RT-PCR primers

Transcript	GenBank/Solea-DB* accession no.	Direction	Primer (5′ to 3′)
lhcgrba	GQ472140	Forward	TCACAGGGGCTGGAGTCAGT
		Reverse	ATCTGTTTGGGGATGGGGAT
fshra	GQ472139	Forward	ggcgactggactgagtttcg
		Reverse	GTGTCTGTAAAGGTTCTCAG
rsph1	KF268356	Forward	Ctggctccacctgaagtctc
		Reverse	GAGACTTCAGGTGGAGCCAG
amh	KF268355	Forward	GGCCCTGTCTCCTGCTCTAT
		Reverse	CGTGCATGTACGACTCCAAG
3bhsd	FJ786643	Forward	ACCTCATCGGAGGGAAGTTT
		Reverse	GAGGACACGGTCCATTGTCT
odf2l	Unigene_237047	Forward	GACGACGCTCAGAGACAGGT
		Reverse	TGCTCTTTGCTTCTCTGCTG
sept7a	Unigene_6151	Forward	CATCTGCCAATCAAACTCCA
		Reverse	CCTGACCCAAACAACAGGAC
cull3	Unigene_64499	Forward	CGAACTCAAAAGTTGGCACA
		Reverse	CCGATTTTGTTTTGTGTGCAT
efa1	AB326302	Forward	GATTGACCGTCGTTCTGGCAAGAAGC
		Reverse	GGCAAAGCGACCAAGGGGAGCAT
bactin	DQ485686	Forward	acatggagaagatctggc
		Reverse	TCCGTAAGGATCTGCATCG

*Solea-DB database v2.0 (www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/sessions/new).