Supplementary Materials

The Ly6/uPAR protein Bouncer is necessary and sufficient for species-specific fertilization

Authors

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Materials and methods

Fish lines and husbandry

Zebrafish (*Danio rerio*) were raised according to standard protocols (28°C water temperature; 14/10 hour light/dark cycle). TLAB fish, generated by crossing zebrafish AB and the natural variant TL (Tupfel Longfin) stocks, served as wild-type zebrafish for all experiments. *Bouncer* mutant zebrafish and transgenic lines were generated as part of this study and are described in detail below. The zebrafish line tg[*actb2:lyn-tdTomato*] was kindly provided by the Heisenberg lab and used for membrane labeling. Wild-type medaka fish (*Oryzias latipes,* strain CAB) were raised according to standard protocols (28°C water temperature; 16/8 hour light/dark cycle) in the fish facility of the Max F. Perutz Laboratories Vienna, and were kindly provided by the Tessmar-Raible lab.

All fish experiments were conducted according to Austrian and European guidelines for animal research and approved by local Austrian authorities (animal protocol GZ: 342445/2016/12 for work with zebrafish; animal protocols BMWFW-66.006/0012-WF/II/3b/2014, BMWFW-66.006/0003-WF/V/3b/2016 and GZ: 198603/2018/14 for work with medaka).

RNA-seq from the zebrafish germline

Testes, ovaries, and mature oocytes (obtained by squeezing) of wild-type (TLAB) zebrafish were collected (two biological replicates for each sample). Total RNA was isolated using the standard TRIzol (Invitrogen, Waltham, MA) protocol, and genomic DNA was removed by TURBO DNase treatment followed by phenol/chloroform extraction. Strand-specific libraries for 76-bp paired-end sequencing were prepared from cDNA by the Broad Institute Sequencing Platform. Libraries were sequenced on the Illumina HiSeq 2000. Reads were aligned to GRCz10, using the Ensembl transcriptome release 88. A custom file was generated by adding *bouncer* based on its position coordinates (exon = chr18:50858259-50858859 (- strand); CDS = chr18:50858285-50858663 (- strand)). The following command was used to map each sample: ‘tophat - o <output directory> -

p 16 --library-type fr-firststrand –no-novel-juncs –g 1 –G <Custom_gene_table> <Bowtie2_genome_index> <fastq_reads>". Quantification of transcript levels was determined using cuffnorm with the following command "cuffnorm -p 22 --library-type=fr-firststrand -L < labels > -o <output_directory> <Custom_gene_table> <aligned_reads.bam file>. Coverage tracks were displayed in the Integrative Genomics Viewer (IGV) (http://software.broadinstitute.org/software/igv/). The RNA-seq data set was deposited to Gene Expression Omnibus (GEO) and is available under GEO acquisition number GSE111882.

Protein domain analysis of Bouncer

Protein domains were assigned using hmmscan (v. 3.1b2) (*34*) and profile hidden Markov models derived from PFAM (v. 31.0, March 2017) (*35*), with a significant E-value threshold of 0.01. The domain search classified zebrafish Bouncer as a member of the Ly6/uPAR protein family (Pfam domain UPAR_LY6_2, E-value 1.4e-05). The conserved domain covers nearly the entire protein (residues 17-100), apart from the amino terminal signal peptide (predicted signal peptide cleavage site after amino acid 18 (VLP-QG) based on SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (*36*)) and a putative transmembrane region at the carboxy terminus (amino acids 109-125, based on TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html) (*37*)). Based on GPI prediction tools (http://mendel.imp.ac.at/gpi/gpi_server.html) (*38*), zebrafish Bouncer is a GPI-anchored protein (GPI anchor site: N98; p-value 2.1e-03). Glycosylation site prediction tools (http://www.cbs.dtu.dk/services/NetNGlyc/ and http://www.cbs.dtu.dk/services/NetOGlyc/) identified two possible N-glycosylation sites in zebrafish Bouncer: amino acids N32 and N84.

Homolog identification and phylogenetic analysis

An NCBI-BLASTP search (version 2.6.0+) (*39*) with *Danio rerio* Bouncer (NCBI gene *LOC101885477*; XP 005173770.1; gene ID:101885477) against the human proteome (NCBI version 12/2017, 357531 non-redundant entries) identified only the SPACA4 protein as a hit (E-value 9.72e-06). A reciprocal search with human SPACA4 protein against the zebrafish proteome (60215 non-redundant entries) returned Bouncer as the best hit (E-value 1.43e-04).

An NCBI-BLASTP search with *Danio rerio* Bouncer against the NCBI non-redundant (nr) protein database (version 11/2017, 138830414 entries) resulted in highly significant hits (below E-value 1e-10) for proteins from bony fishes such as *Oncorhynchus mykiss* (rainbow trout), *Hippocampus comes* (tiger tail seahorse) and *Oreochromis niloticus* (Nile tilapia). The closest hits outside of bony fish were for SPACA4 (sperm acrosome membrane-associated protein 4) family proteins in *Myotis brandtii* (Brandt's bat, E-value 1.20e-09) and *Xenopus tropicalis* (tropical clawed frog, E-value 5.09e-09). Since no medaka (*Oryzias latipes*) Bouncer could be detected in the NCBI nr database, we performed a TBLASTN search within the NCBI EST division and identified the cDNA clone McF0051J12-MGRbd1 (NCBI EST accession AM154129) as the best hit (E-value 3e-15), coding for a 131-amino acid gene product. The homolog relationship was confirmed in a reciprocal BLAST search against the zebrafish genome and in a phylogenetic tree with selected members of the Ly6/uPAR family (see below).

Phylogenetic tree

Starting with *Danio rerio* Bouncer, we collected all significant (E-value 0.001) hits in an NCBI-BLASTP search within the non-redundant protein database. To extend the Ly6/uPAR family, these hits were used as a query in an additional search step applying significant criteria (E-value 0.001) for hit collection. Forty-two species were selected to represent a wide taxonomic range. Within each species, the sequences were reduced to 80% identity with cd-hit (*40*). Proteins were aligned with mafft (-linsi v7.313) (*41*), and sequences with long gaps were removed. The medaka (*Oryzias latipes*) Bouncer sequences and one Ly6/uPAR protein of the shark *Squalus acanthias* were added (see Supplemental Table 1) and realigned, resulting in a final set of 192 full-length sequences. The respective coding sequences were extracted from the NCBI website (where available, see table S1), and aligned with RevTrans using the protein sequence alignment (v. 1.4) (*42*). Aligned columns with less than 50% sequence information were removed. The alignment was furthermore restricted to the region corresponding to the putative mature peptide of *Danio rerio* Bouncer using Jalview (*43*). A maximum likelihood phylogenetic tree was calculated with IQ-TREE (v. 1.5.5) (*44*) with codon model selection using ModelFinder (*45*) and 1000 ultrafast bootstrap replicates (*46*). The tree was visualized in iTOL (*47*).

Analysis of the evolutionary divergence of fish Bouncer proteins

To assess whether the evolutionary divergence of fish Bouncers is comparable to the evolutionary divergence of known mammalian fertilization factors, we calculated the percentile rank of (1) putative orthologous fish Bouncers (using *Danio rerio* Bouncer protein as reference) and (2) putative orthologous fertilization factors from placental mammals (using human proteins as references). To this end, UNIPROT reference proteomes were searched, including the additional dataset with isoforms and variants; the proteomes from *Danio rerio* and *Homo sapiens* served as reference proteomes, and in the case of *Danio rerio*, Bouncer was added, since it is not present in the initial data set. Search parameters were as follows: NCBI-BLASTP with default E-value threshold of 10; the hitlist was limited to 20 target sequences. Only hits that were equal or below an E-value threshold of 0.01 were considered for the reciprocal hitlist. An ortholog relationship was assigned when the best hit of a query protein (of the reference proteome) to the target proteome matched as the best hit in a reciprocal blast the same geneID of the query protein in the reference proteome. For each geneID of the reference proteome, the percent identity of the high-scoring pair (HSP) with the lowest E-value in the orthologous reciprocal blast was taken. The percent identity was then used to calculate the percentile rank of this orthologous protein pair in relation to all other orthologous pairs identified for these two species. For example, Medaka Bouncer has a percent identity to zebrafish Bouncer of 37.9% within the HSP; this 37.9% corresponds to a rank of 262 out of 13389 pairwise orthologs (equivalent to a percentile rank of 2.0%) that could be identified between these two species (sorted from lowest to highest percent identity).

Generation of *bouncer* knockout fish

Bouncer mutant fish were generated by Cas9-mediated mutagenesis, targeting the predicted (*9*), un-annotated single-exon gene downstream of the *ppfia1* gene (location of the *bouncer* ORF in GRCz10: chr18:50,858,463-50,858,837). A guide RNA (sgRNA) targeting the region encoding the N-terminal signal peptide of Bouncer was generated according to published protocols (*48*) by

oligo annealing followed by T7 polymerase-driven *in vitro* transcription (gene-specific targeting oligo: bouncer_gRNA; common tracer oligo). Cas9 protein and *bouncer* sgRNA were co-injected into the cell of one-cell stage TLAB embryos. Putative founder fish were outcrossed to TLAB wild-type fish. A founder fish carrying a germline mutation in the *bouncer* gene was identified by a size difference in the *bouncer* PCR amplicon in a pool of embryo progeny (primer: bouncer_gt_F and bouncer_gt_R). Embryos from this founder fish were raised to adulthood. Amplicon sequencing of adult fin-clips (primers: bouncer_gt_F and bouncer_gt_R) identified the mutation, a 13-nt deletion, which introduces a premature termination codon at the end of the Bouncer signal peptide sequence.

Genotyping of *bouncer* mutant fish was performed by PCR (primers: bouncer_gt_F and bouncer_gt_R). Detection of the 13-nt deletion was performed by standard gel electrophoresis using a high-percentage agarose gel either a) without further processing of the PCR product, or b) after EcoNI digest, which cuts only the wild-type but not the mutant amplicon (wild-type amplicon: 234-nt, 13-nt deletion amplicon: 221-nt, EcoNI-digested wild-type amplicon: 126-nt + 108-nt).

Homozygous *bouncer*$^{-/-}$ knockout fish (*bncr*$^{-/-}$*)* were generated by incrossing *bncr*$^{+/-}$ fish or by crossing a *bncr*$^{-/-}$ male to a *bncr*$^{+/-}$ female. Alternatively, *bncr*$^{-/-}$ fish were generated by crossing a *bncr*$^{-/-}$ female, carrying a sfGFP-tagged or untagged *bouncer* rescue construct (*tg[ubi:sfGFP-bncr, cmlc2:eGFP]* or *tg[ubi:bncr, cmlc2:eGFP]*), to a *bncr*$^{-/-}$ male and screening for the absence of green fluorescent hearts.

Generation of zebrafish expressing transgenic Bouncer

The predicted *bouncer* transcript unit, including its 24-nt 5'UTR, 378-nt ORF encoding the 125-amino acid zebrafish Bouncer protein, and 236 nts of its 3'UTR, was amplified by PCR from cDNA derived from 2-4 cell stage zebrafish embryos (primers: bouncer_F; bouncer_R) and cloned into the pSC vector (Strataclone) to generate *pSC-bncr*. sfGFP (superfold GFP)-tagged Bouncer (*pSC-signalpeptide-sfGFP-bncr*) was generated by digesting *pSC-bncr* with EcoNI, which cuts *bncr* at the end of the predicted signal peptide sequence (...VVLP-Q…), and by inserting a sfGFP-encoding PCR fragment (primers: sfGFP-bouncer_F; sfGFP-bouncer_R) in frame immediately after the predicted signal peptide sequence (…VVLP-sfGFP…) at the N-terminus of the mature Bouncer protein by Gibson cloning (*49*). Untagged *bouncer* and *sfGFP-tagged bouncer* were subsequently amplified by PCR and subcloned into vectors for Tol2-based transgenesis (BamHI/NheI-cut *pTol2-ubi:MCS, cmlc2:GFP* or BamHI/AgeI-cut *pMTB-actb2:H2B-Cerulean* (kind gift from Sean Megason)) to generate *pTol2-ubi:bncr, cmlc2:eGFP; pTol2-ubi:sfGFP-bncr, cmlc2:eGFP;* and *pMTB-actb2:sfGFP-bncr.*

To generate sfGFP-tagged Bouncer with mutations of the two predicted N-glycosylation sites (N32, N84), a gBlock (SH043; IDT) encoding part of the sfGFP ORF and the N32A/N84A (AAC -> GCC) mutated *bouncer* ORF was cloned into ClaI/XmaI-digested *pTol2-ubi:sfGFP-bncr, cmlc2:eGFP* to obtain *pTol2-ubi:sfGFP-bncr*$^{N32A,\ N84A}$*, cmlc2:eGFP*.

To generate secreted sfGFP-tagged Bouncer lacking the GPI anchor site and C-terminal transmembrane domain (sfGFP-Bouncer^{noTM}), a gBlock (SH044, IDT) encoding the truncated *bouncer* ORF was cloned into ClaI/XmaI-digested *pTol2-ubi:sfGFP-bncr, cmlc2:eGFP* to obtain *pTol2-ubi:sfGFP-bncr*^{noTM}*, cmlc2:eGFP*.

To generate transgenic zebrafish lines expressing medaka Bouncer, medaka Bouncer (NCBI nucleotide accession number AM154129.1) was PCR-amplified from cDNA derived from oocyte mRNA (primers: medaka_bouncer_F and medaka_bouncer_R) and cloned into a vector for Tol2-based transgenesis (BamHI/NheI-cut *pTol2-ubi:MCS, cmlc2:eGFP*) *to obtain pTol2-ubi:medaka-bncr, cmlc2:eGFP.*

Zebrafish lines expressing transgenic Bouncer were generated by injecting *bouncer* expression constructs with *Tol2* mRNA into TLAB or *bncr*$^{+/-}$ zebrafish embryos (4 ng/µl of medaka Bouncer plasmid or 15 ng/µl of all other purified plasmids in RNase-free water, 9.3 ng/µl for medaka Bouncer transgenesis or 35 ng/µl *Tol2* mRNA for all others, 0.083% phenol red solution (Sigma-Aldrich)), following standard procedures. Injected embryos with high expression of the fluorescent marker (either *cmlc2:eGFP*, or *actb2:sfGFP-bncr*) at one day post fertilization were raised to adulthood. Putative founder fish were crossed to *bncr*$^{-/-}$ or *bncr*$^{+/-}$ fish, and the progeny was screened after 48 hours for the expression of the fluorescent marker. GFP-positive embryos were then raised to adulthood and genotyped for *bouncer*. Functionality of the expression constructs was determined by assessing the ability to rescue the near-sterility of *bncr*$^{-/-}$ females.

The following transgenic zebrafish lines were generated (in wild-type (TLAB), *bncr*$^{+/-}$ or *bncr*$^{-/-}$ backgrounds):

- *tg[ubi:bncr, cmlc2:eGFP]*
- *tg[ubi:sfGFP-bncr, cmlc2:eGFP]*
- *tg[actb2:sfGFP-bncr]*
- *tg[ubi:sfGFP-bncr*^{noTM}*, cmlc2:eGFP]*
- *tg[ubi:sfGFP-bncr*$^{N32A,\ N84A}$*, cmlc2:eGFP]*
- *tg[ubi:medaka-bncr, cmlc2:eGFP]*

The following expression constructs were determined to be fully functional (rescuing the near-sterility *of bncr*$^{-/-}$ females: *ubi:bncr, ubi:sfGFP-bncr, actb2:sfGFP-bncr,* and *ubi:sfGFP-bncr*$^{N32A,\ N84A}$.

Imaging of live embryos

Live embryos and larvae were imaged in their chorions in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% Methylene Blue) using a Stereo Lumar.V12 fluorescence microscope (Zeiss).

Antibodies

To generate an antibody specific for zebrafish Bouncer, two *in vitro*-synthesized peptides (CYADGRFGRSSVLFRKG and CSRSRHQMIRGNNIS) were used for immunization of rabbits (Eurofin). The final bleed was purified against each of the two peptides separately. For this purpose, each peptide was bound to a column by first dissolving 5 mg of the peptide in 3 ml of 20 mM Hepes and 1 mM EDTA. The peptide solution was then mixed with 300-310 mg maleimide-activated POROS and flushed with argon. Afterwards, the suspension was incubated at RT for 1 hour with gentle mixing. The column (4.6 x 50 mm) was packed using column packer (Applied Biosystems, buffer: HBS; flow: 10 ml/min for 20-25 min) on HPLC. For the affinity purification (monitoring at 280 nm, flow: 7.5 ml/min), 5 ml of the serum were injected onto the column, followed by a wash with HBS. For the first elution $MgCl_2$-containing buffer (1.5 M $MgCl_2$, 50 mM NaAc, pH 5.2), and for the second elution glycine buffer (0.1 M glycine, 0.1 M NaCl pH 2.45) were used.

The purified antibody was dialyzed using HBS buffer and stored in 10% glycerol at 4°C. The different antibody fractions were tested for their reactivity against Bouncer by western blotting. The glycine eluate purified against peptide CYADGRFGRSSVLFRKG gave the strongest and cleanest signal for Bouncer and was therefore used for all experiments.

Analysis of zebrafish egg and embryo lysates by western blotting

For western blotting of zebrafish egg and embryo lysates, freshly laid eggs or dome stage embryos (as indicated) were dechorionated manually in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% Methylene Blue). Afterwards, the yolk was manually removed using forceps, and the egg/embryo caps were frozen in liquid nitrogen and stored at -80°C until usage. Western blotting was performed following standard protocols. In brief, the equivalent of 20 eggs (or embryos) were loaded per lane. SDS-PAGE was performed using Mini-PROTEAN® TGX™ Precast Protein Gels or 10–20% Mini-PROTEAN® Tris-Tricine Gels. Blotting was performed using a wet-blot system (BioRad). The following antibodies were used in western blotting: anti-Bouncer, rabbit, 1:500 (generated as described above), anti-tubulin (mouse, Sigma-Aldrich T6074, 1:20000).

Analysis of glycosylation of Bouncer

Manually deyolked egg and embryo samples were deglycosylated overnight under non-denaturing conditions using Protein Deglycosylation Mix (New England Biolabs) according to the manufacturer's protocol. The glycosylation state of endogenous Bouncer was assessed by western blotting using Bouncer-specific antibodies. Twenty one-cell-stage embryos, which were manually dechorionated and deyolked, were loaded per lane.

***In situ* hybridization**

Bouncer DIG-labeled RNAs (sense and antisense) were generated by digesting *pSC-bncr* with EcoRV (antisense) or BamHI (sense), followed by *in vitro*-transcribing the linearized plasmids using T7 (antisense) or T3 (sense) polymerases (Roche) and DIG RNA labeling mix (Roche). *In situ* hybridization in zebrafish embryos was performed according to standard protocols, using *anti-bouncer* or *sense-bouncer* (negative control for nonspecific staining) DIG-labeled RNA probe. BCIP/NBT/alkaline-phosphatase-stained embryos were dehydrated in methanol and imaged in BB/BA using a Stereo Lumar.V12 fluorescence microscope (Zeiss) and an Axioplan 2 microscope (Zeiss) equipped with a DFC320 camera (Leica).

***In vivo* fertilization in fish**

The evening prior to mating, male and female fish were separated in breeding cages (one male and one female per cage). The next morning, male and female fish were allowed to mate by removing separators. Eggs were collected from the bottom of the breeding cages and kept at 28°C in E3 medium. The rate of fertilization was assessed about 3 hours post laying. By this time, fertilized embryos have developed to ~1000-cell stage embryos, while unfertilized embryos resemble one-cell stage embryos.

In vitro fertilization in fish

In vitro fertilization experiments were performed following standard procedures (*50*, *51*). The evening prior to the zebrafish egg and sperm collections, male and female zebrafish were separated in breeding cages (one male and one female per cage).

To collect mature, un-activated eggs, female zebrafish were anesthetized using 0.1% w/v tricaine (25x stock solution in dH_2O, buffered to pH 7-7.5 with 1 M Tris pH 9.0). After being gently dried on a paper towel, the female was transferred to a dry petri dish, and eggs were carefully expelled from the female by applying mild pressure on the fish belly with a finger and stroking from anterior to posterior. The eggs were separated from the female using flat forceps or a small paintbrush, and the female was transferred back to the breeding cage filled with fish water for recovery.

To collect un-activated zebrafish sperm, male zebrafish were anesthetized using 0.1% tricaine. After being gently dried on a paper towel, the male fish was placed belly-up in a slit in a damp sponge under a stereomicroscope with a light source from above. Sperm was collected into a glass capillary by mild suction while gentle pressure was applied to the fish's belly using flat forceps. The sperm of several males (2–5) was stored in ice-cold Hank's saline (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) on ice. The male was transferred back to the breeding cage filled with fish water for recovery. To label sperm with MitoTracker, sperm was incubated in 0.5 µM MitoTracker^{TM} Deep Red FM (Thermo Fisher Scientific) on ice for >10 minutes prior to *in vitro* fertilization.

Un-activated medaka sperm was collected according to published procedures (*51*). The sperm was stored in ice-cold Hank's saline (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) on ice until usage. Activation of sperm from both species was confirmed upon addition of water by visual inspection of their motility at 40x magnification. Medaka sperm was demonstrated to fertilize wild-type medaka eggs under the described conditions.

To fertilize eggs *in vitro*, an aliquot of 25-50 µl unlabeled or MitoTracker-labeled sperm solution was added to eggs of potentially different genotypes (e.g. collected from wild-type, *bncr*$^{-/-}$ or transgenic females) in individual petri dishes. Sperm addition was immediately followed by adding 500 µl-1 ml of E3 medium to simultaneously activate sperm and eggs. After 2 minutes, petri dishes were filled with E3 medium and kept at 28°C. The rate of fertilization was assessed about 3 hours post sperm addition. By this time, fertilized embryos have developed to ~1000-cell stage embryos, while unfertilized embryos resemble one-cell stage embryos.

Analysis of egg activation and polar body extrusion

Egg activation was assessed by monitoring chorion elevation after addition of E3 medium to un-activated eggs that had been obtained by squeezing.

To assess polar body extrusion, eggs were *in vitro* fertilized and fixed after 3, 10 and 20 minutes in 3.7% formaldehyde in PBS at 4°C overnight. Embryos were washed in PBST, permeabilized in methanol and kept at -20°C. For immunostaining, embryos were transferred back to PBST, dechorionated manually using forceps, and stained with a rabbit anti-γ-tubulin antibody (T3559 Sigma, used at 1:1000; secondary antibody: goat anti-rabbit Alexa488 (A-11034 Thermo Scientific, used at 1:250)) and 1x DAPI in PBS. Embryos were mounted in 1% low-melt agarose on glass-bottom dishes (Ibidi) and imaged at an inverted LSM880 confocal microscope (Zeiss) with a 20x objective lens at 2x magnification.

Analysis of cytoplasmic streaming

Embryos derived from *in vivo* mating crosses between wild type males and *wild-type* or *bncr*$^{-/-}$ females were collected immediately after being laid and dechorionated with pronase (1 mg/ml). Dechorionated embryos were injected with 1 nl of red fluorescent FluoSpheres® NeutrAvidin® labeled microspheres (ThermoFisher, 1:5 dilution in 0.083% phenol red) in the lower third of the yolk. Directly after injection, embryos were mounted laterally in glass-bottom dishes (Ibidi) in 0.8% LM (low-melt) agarose in PBS. Embryos were imaged using an inverted LSM 780 confocal microscope (Zeiss) with a 10x objective lens at 1x magnification for 200 cycles over 3 hours. Embryos were kept at 28°C during imaging.

Statistical analysis was performed using Prism software (Graphpad). Small accumulations of beads were tracked (on average, 8 bead accumulations per embryo) using the manual tracking function in Fiji (*52*), which recorded the velocity for each time point. The mean velocity that each bead traveled in each embryo was calculated; these values were then averaged to produce the total mean velocity of all beads in a single embryo. An unpaired t-test was used to compare the total mean velocities between wild type and *bouncer* knockout embryos.

Scanning electron microscopy

Eggs from wild-type and *bncr*$^{-/-}$ females were squeezed into dry petri dishes. E3 medium was added for activation, and after 10 seconds to 1 minute, glutaraldehyde (2.5%) was added for initial fixation. The samples were then stored at 4°C overnight. The eggs were transferred to 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 and incubated for 2 hours. Eggs were rinsed with the same buffer and post-fixed in 1% osmium tetroxide in ddH_2O. After rinsing with ddH_2O, samples were dehydrated by a 10-minute treatment in dimethoxipropane followed by 3 incubation steps in anhydrous acetone for 30 minutes each. The eggs were then treated with a 1:1 mixture of anhydrous acetone and hexamethyldisilazane (HMDS) followed by 3 HMDS treatments for one hour each. After air-drying for several hours in a fume hood, eggs were gold sputter coated and examined in a Hitachi TM-1000 tabletop scanning electron microscope operated at 15 kV and equipped with a highly sensitive semiconductor backscattered electron detector.

Coomassie staining of the micropyle

To stain the micropylar region of the chorion, eggs from normal *in vivo* crosses were collected ~10 minutes after egg laying and fixed in 3.7% formaldehyde in PBS at 4°C overnight. Embryos were washed in PBST and stained with Coomassie Brilliant Blue G (final concentration: 0.2% in 10% DMSO in PBS) for 10 minutes at RT. Embryos were rinsed in PBS. The chorion was manually separated from the embryo using forceps, and flat-mounted on a glass slide. Images of the micropylar region were taken with bright field optics using an Axio Imager.Z1 microscope (Zeiss) with 20x or 40x objectives. Quantification of the diameter of the micropylar region was performed in FIJI, using the measurement tool. The average diameter of each micropyle was calculated from two perpendicular measurements of each micropylar opening. Statistical analyses were performed using Prism software (Graphpad). Unpaired t-tests were used to compare the average diameters of the micropyles between wild-type and *bouncer* knockout embryos.

Intra-cytoplasmic sperm injection (ICSI) in zebrafish

Sperm of two males was pooled in 50 µl of Hank's saline (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$). To assess the sperm concentration, a small aliquot of sperm was diluted 1:100 in Hank's saline, and the sperm number was counted using a cell counting chamber (Neubauer pattern, 0.1 mm depth, 0.0025 m^2, BRAND®) on a cell culture microscope (Axio, Vert.A1, Zeiss). The sperm solution was diluted to a working concentration of 2000–4000 sperm/µl in 5% polyvinylpyrrolidone (PVP) in E3 medium and filled into the injection needle using Microloader™ pipette tips (Eppendorf). The sperm number was checked by injecting 0.5 nl of the sperm mix into seven 2-µl droplets of a 1x DAPI solution containing 10 mg/ml digitonin in 5% PVP, using a standard microinjection apparatus (PV820 Pneumatic PicoPump, World Precision Instruments). The sperm in the droplets was imaged using a cell culture microscope (Axio, Vert.A1, Zeiss). If the sperm number was correct (1-2 sperm/0.5 nl), un-activated, mature eggs were collected from a female and kept in Hank's saline (+ 0.5% BSA) during injection to slow down egg activation. During injection, eggs were held in place in 1.5% wedge-shaped agarose wells in a petri dish with the help of flat forceps. Sperm was injected into the cell as close as possible to the micropyle. After injection, the eggs were immediately transferred to E3 medium and kept at 28°C. After 3–5 hours, the injected eggs were analyzed for cell cleavage.

Analysis of Bouncer localization during egg activation

To analyze the localization of Bouncer in zebrafish eggs during activation, un-activated, mature eggs were collected from female fish expressing sfGFP-Bouncer as well as membrane-bound lyn-Tomato (tg[*ubi:sfGFP-bncr, cmlc2:GFP*]; tg[*actb2:lyn-Tomato*]) by squeezing. Eggs were mounted in a petri dish with cone-shaped agarose molds (1.5% agarose in E3 medium) filled with Hank's saline (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) to slow down activation. Imaging was performed at a LSM700 Axio Imager upright system (Zeiss) with a 40x0.75 Archoplan dipping lens at RT.

Imaging of sperm approach

Sperm was squeezed from 2–4 wild-type male fish and kept in 150 µl Hank's saline containing 0.5 µM MitoTracker™ Deep Red FM (Molecular Probes) for >10 minutes on ice. Un-activated, mature eggs were obtained by squeezing a female wild-type or *bncr*$^{-/-}$ fish. To prevent activation, eggs were kept in sorting medium (Leibovitz's medium, 0.5 % BSA, pH 9) (*53*) at RT. The eggs were kept in place using a petri dish with cone-shaped agarose molds (1.5% agarose in sorting medium) filled with sorting medium. Imaging was performed at a LSM700 Axio Imager upright system (Zeiss) with a 10x/0.3 Archoplan dipping lens (781.96 msec interval, for 1–5 minutes). To start fertilization, most of the sorting medium was removed without letting the dipping lens go completely dry and at least 1 ml of E3 medium was carefully added close to the egg in order to induce activation. Immediately afterwards, 10 µl of the stained sperm was added as close to the egg as possible. The resulting time-lapse movies were analyzed using FIJI.

Analysis of sperm-egg binding

Sperm was squeezed from 2–4 male fish and kept in 100 µl Hank's saline + 0.5 µM MitoTracker™ Deep Red FM on ice. Un-activated, mature eggs were squeezed from a wild-type or *bncr*$^{-/-}$ female

fish and activated by addition of E3 medium. After 10 minutes, 30 wild-type or Bouncer-deficient eggs were manually dechorionated using forceps. The dechorionated eggs were transferred to a glass dish and incubated at room temperature for > 1 hour. To enable sperm binding, the glass dish was tilted and as much E3 medium as possible was removed without letting the eggs become dry. Sperm solution (45 µl) was distributed onto each egg batch and the sperm/egg mixture was incubated for another 2 minutes. The glass dish was then carefully filled with E3 medium and the eggs were transferred to a zebrafish transplantation mold (Adaptive Science Tools) (1.5% agarose in blue water) to hold them in place during imaging. Imaging was performed at a LSM700 Axio Imager upright system (Zeiss) with a 10x/0.3 Archoplan dipping lens. Sperm attached to the cell of the egg were counted during live imaging. If possible, single sperm were counted (1–10 sperm); if more than 10 sperm were attached to the egg, the egg was classified as >10 sperm bound.

qRT-PCR analysis of medaka Bouncer expression levels

To assess the expression levels of medaka *bouncer*, qRT-PCR was performed using three biological replicates of 25 unfertilized eggs squeezed from each transgenic medaka Bouncer female tested in *in vitro* fertilization experiments (except for female 1, for which only one sample was obtained). RNA was isolated using the standard TRIzol (Invitrogen, Waltham, MA) protocol followed by phenol/chloroform extraction. cDNA was synthesized using the iSCRIPT cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Levels of medaka *bouncer* mRNA were quantified using primers specific for the transgene sequence (medakabncr_qF and medakabncr_qR) in qRT-PCR with GoTaq qPCR Master Mix (Promega, Madison, WI). The following program was run: 10 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C, ending with 1 min at 95°C. Medaka *bouncer* mRNA expression levels were normalized to *beta-actin* transcript levels (primers: b-actin_qF and b-actin_qR).

Analysis of gDNA content in zebrafish, medaka and hybrids

Zebrafish, medaka, and hybrid gDNA was isolated according to standard protocols (*54*). In brief, a single embryo at sphere stage (zebrafish, hybrids) or stage 11-12 (medaka) was transferred into 100 µl 50 mM NaOH and incubated for 20 min at 95°C (the medaka embryos were disrupted with a pipette tip prior to incubation). The samples were cooled to 10°C, and 1 µl of the isolated gDNA was used for touchdown PCR (protocol: 95°C for 2 min, followed by 15 cycles of: 95°C for 30 sec, 70°C for 30 sec (-1 °C each cycle), 72°C for 40 sec, followed by 20 cycles of: 95°C for 30 sec, 55°C for 30 sec, 72°C for 40 sec, followed by 72°C for 5 min and hold at 10°C) (*55*) using Taq polymerase and species-specific primers (zebrafish: z-vox, z-klf4; medaka: m-vox, m-klf4).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 software. Statistical significance of differences between two experimental groups was assessed wherever applicable by either a two-tailed Student's t-test if the variances were not significantly different according to the F test, or using a non-parametric test (Mann-Whitney or Kruskal-Wallis with Dunn's test for multiple comparisons) if the variances were significantly different ($p < 0.05$). Differences between two data sets were considered significant at $p < 0.05$ (*) ($p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****); n.s., not significant. Linear regression analysis was performed to determine the goodness of fit

for a linear equation relating the trend between fertilization rate and transgene expression with 95% confidence intervals.

Primer sequences

name of oligo	sequence	comments
bouncer_gRNA	taatacgactcactataGGCAGCGGAGACCCTGAGGGgttttagagctagaaatagcaag	t7 promoter sequence - gRNA TARGETING SEQUENCE – tracerOligo annealing sequence
tracer_oligo	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Common oligo used to generate guideRNAs by annealing and in vitro transcription (Gagnon et al., 2014)
bouncer_gt_F	TCTTTCCCTACACGACGCTCTTCCGATCTCagctgctcatcctcctcatg	Genotyping of *bouncer*: the DNA binding region is marked by lower case letters.
bouncer_gt_R	TGGAGTTCAGACGTGTGCTCTTCCGATCTaccggacactcggtcactac	
bouncer_F	TCAGTGTGTGTGTTCTGCTTCAGCATGGGCTGTGTGTTGCTGTT	to clone *bncr* ORF including 5'UTR and 3'UTR into pSC
bouncer_R	TTAAAATTCTCCGATAGTTTAATGAGTTC	
sfGFP-bouncer-F	TGCTGCTGGTGTGTGTCCCGGTgtcctccctGTGTCTAAAGGAGAGGAGCT	to introduce sfGFP tag into EcoNI cut *pSC-bncr*; template: *pCS2+sfGFP* (kindly provided by Jeff Farrell)
sfGFP-bouncer-R	ACCGGGCAGAAGAGGCAGCGGAGACCctgaggTTTGTACAGCTCATCCATGC	
bouncer-BamHI_F	gctgggatccTCAGTGTGTGTGTTCTGCTTCAGCATGGGCTGTGTGTTGCTGTT	to subclone *bncr* (tagged or untagged) with 5'UTR and 3'UTR into BamHI/NheI-cut *pTol2-ubi:MCS, cmlc2GFP*
bouncer-XbaI_R	gccagtctagaTTAAAATTCTCCGATAGTTTAATGAGTTC	
bouncer-BamHI_Gibson_F	gagaagctacttgttctttttgcaGGATCCgccaccATGGGCTGTGTGTTGCTGTTTCTG	to subclone sfGFP-tagged *bncr* with 5'UTR and 3'UTR into BamHI/AgeI-cut *pMTB-actb2:H2B-Cerulean* (kindly provided by Sean Megason), thereby exchanging *H2B-Cerulean* for *sfGFP-Bouncer*
bouncer-AgeI_Gibson_R	gtatcttatcatgtctggatcaaccggtTTCTAGATTAAAATTCTCCGATAG	
medaka_bouncer_F	aatcttactttgaatttgtttacagggatccAGCAACTTCCACTGGCAGGAA	to clone the medaka *bncr* ORF into BamHI/NheI-cut *pTol2-ubi:MCS, cmlc2GFP*
medaka_bouncer_R	atcatcatcgtaccatggtttgctagcTCATGTTAGACAAAAGCTGCAGGC	
z-vox_F	GGTCATTCCCGTTCAGTATCAATG	Zebrafish-specific amplification of vox to analyze the presence of zebrafish gDNA in hybrid embryos
z-vox_R	GATGTTGAGGATGAGGATGGTGAG	
m-vox_F	GTCGAGTGCGCACAAAGTTTAC	Medaka-specific amplification of vox to analyze the presence of medaka gDNA in hybrid embryos
m-vox_R	CCAGCGTCTAAATATTTGTGTTTGC	
z-klf4_F	ATCCTGCGTATCTCCAACCTAC	Zebrafish-specific amplification of klf4 to analyze the presence of zebrafish gDNA in hybrid embryos
z-klf4_R	CGGACACTCCTGCTTTATTCTAGG	

m-klf4_F	TCCTTGATGGAGCTTTATGCG	Medaka-specific amplification of klf4 to analyze the presence of medaka gDNA in hybrid embryos
m-klf4_R	GCCTTGAGATGAGAACTCTTTGTG	
b-actin_qF	ATCAGGGTGTCATGGTTGGT	Used for amplification of beta-actin as a reference gene in zebrafish for qRT-PCR analysis.
b-actin_qR	CACGCAGCTCGTTGTAGAAG	
medakabncr_qF	GAACCAGGCAGCTCTTCCAT	Amplification of medaka Bouncer transgene sequence for qRT-PCR analysis.
medakabncr_qR	TTCGGAGGGCATTCTGTCAC	

gBlock sequence (SH043):
GAACAGAATCGAGCTGAAAGGCATCGATTTCAAAGAGGATGGCAACATCCTGGGACACAA
ACTGGAGTACAACTTCAACAGCCACAACGTGTACATCACAGCCGATAAACAGAAGAACGGC
ATCAAAGCCAACTTCAAGATCAGACACAATGTGGAGGATGGATCTGTGCAGCTGGCCGAT
CACTACCAGCAGAACACACCTATCGGAGACGGCCCAGTGCTGCTGCCAGATAACCACTAC
CTGAGCACACAGAGCGTGCTGTCTAAAGACCCTAACGAGAAGAGAGATCACATGGTGCTG
CTGGAGTTTGTGACAGCCGCCGGAATCACCCTGGGCATGGATGAGCTGTACAAAcctcagGG
TCTCCGCTGCCTCTTCTGCCCGGTAACGTCGCTGgccAGCTCGTGTGCGCCGGTAGTGACC
GAGTGTCCGGTGCAGGAGCTCTGCTACACCGCGGACGGCCGCTTCGGGCGCTCCTCCGT
GCTCTTCCGCAAGGGCTGCATGTTGAGGGCAGACTGCAGCCGCTCCAGACACCAGATGAT
CCGGGGAAACgccATCAGCTTCAGCTTCTCCTGCTGCGGGGGACACTACTGCAACTCCCAG
CCGAGAGCGGAGCCCGGGGGCCGaCTACTGCTGCTGCTGCTG

gBlock sequence (SH044 (noTM)):
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CAACATCAGCTTCAGCTTCTCCTGCTGCGGGGGACACTACTGCTGACGGAGAAacacacacca
gacagacgaataaacacacaccaCACCAgaccaaaccgagacaaacaaacaaacaaacTGTCAGCAGAGTGAA
CATgCTaACACAGCTGCTCCTTCTGGCCGTCATCAAGCGGTCAGAGACAGTGAGCTGGACA
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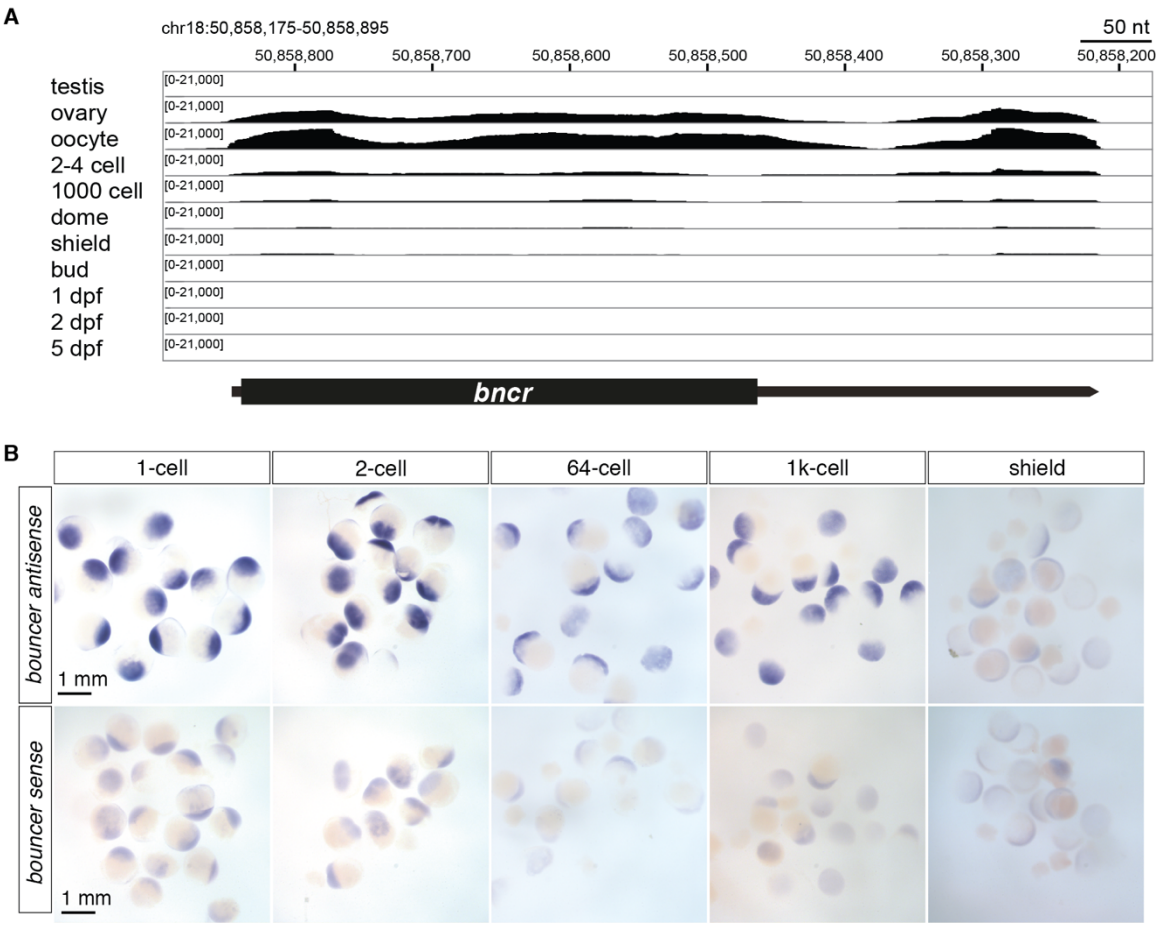

figure S1: Bouncer is maternally provided

A – Expression of zebrafish *bouncer* in the germline and during embryonic development. Coverage tracks for RNA sequencing ((*56*) and this study) show high transcription of the single exon gene *bouncer* in the female germline, and decreasing amounts of *bouncer* RNA upon progression through embryogenesis. Genomic coordinates are based on GRCz10. Dpf, days post fertilization.

B – *In situ* hybridization confirms that *bouncer* is a maternally provided transcript. Shown are representative overview images of *in situ* hybridization using an antisense *bouncer* probe (top) and a sense *bouncer* probe (bottom; negative control for unspecific staining).

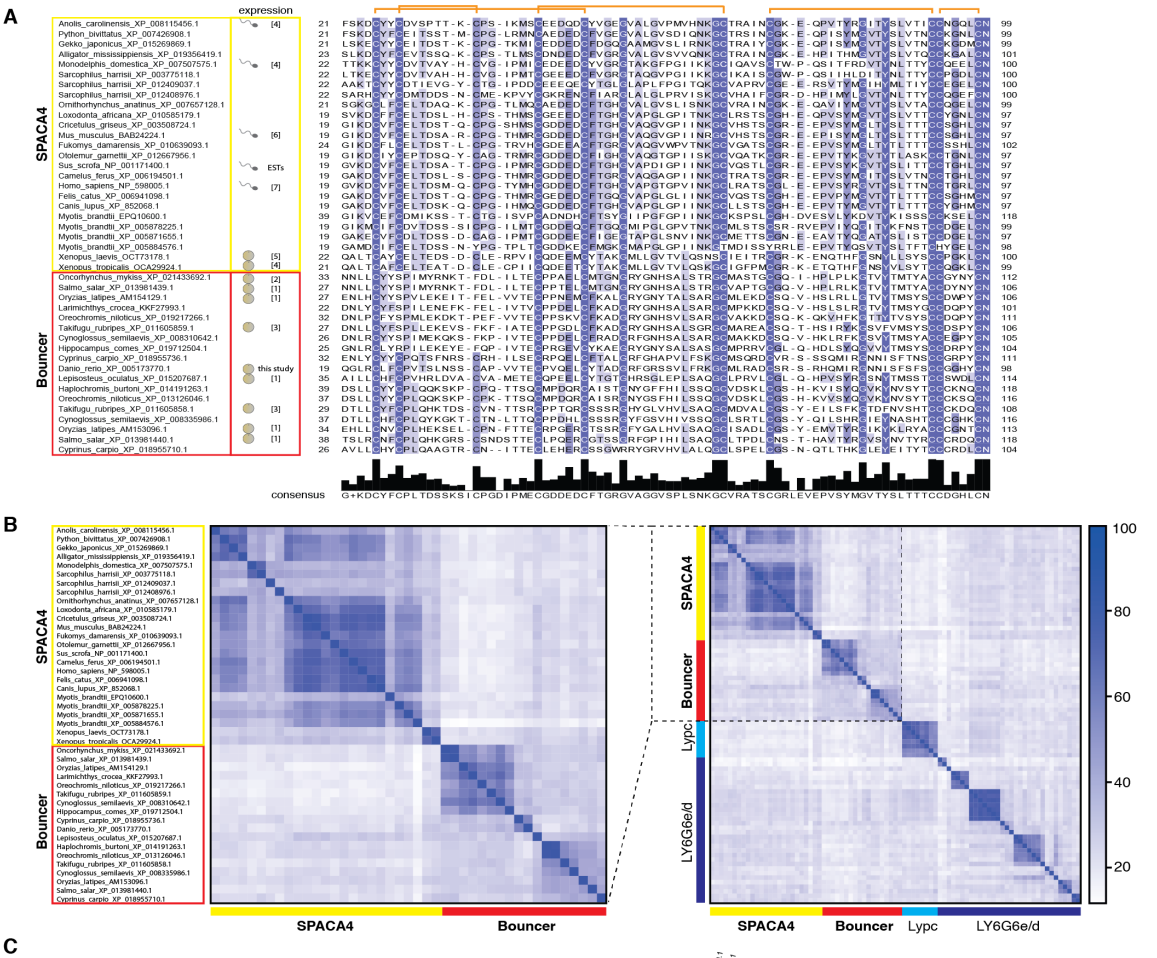
A
expression
SPACA4
Bouncer
ESTs
this study
consensus
B
SPACA4
Bouncer
Lypc
LY6G6e/d
C
Tree scale: 1
CD59
Ly-6
fish
mammals/reptiles/amphibians/fish
Spaca4l
Lypc
LY6G6e/d

figure S2: Bouncer/SPACA4 belongs to the divergent Ly6/uPAR protein family

A – Alignment of representative protein sequences of fish Bouncers and homologous mammalian and reptile SPACA4s shows high sequence divergence between species, apart from the well conserved cysteines. Only amino acid sequences of the conserved mature domain are shown; the extent of the mature domain displayed here is based on the prediction for zebrafish Bouncer. Predicted disulfide bridges are indicated in orange. Sex-specific germline expression is indicated for organisms where expression data is available (sperm: testis-specific expression; egg: ovary-specific expression). (1) (*31*) (RNA-seq data from *Salmo trutta*); (2) (*57*); (3) (*33*); (4) (*30*); (5) (*32*); (6) (*29*); (7) human expression based on ebi-baseline expression atlas (GTEx Portal); ESTs are derived from NCBI. For sequences and accessions see database S1 and S2 and table S1.

B – Bouncer/SPACA4 proteins have divergent amino acid sequences. Left: Heat map showing the percentage amino acid identity between Bouncer and SPACA4 proteins of the subset of vertebrate species shown in A. Right: Heat map showing the percentage amino acid identity between Bouncers, SPACA4, Lypc, and the group of LY6G6e/d proteins.

C – Phylogenetic tree of Ly6/uPAR proteins that are most similar to the SPACA4/Bouncer family. The tree was constructed with a selected subset of vertebrate species (see Materials and Methods for details). Bootstrap values of $\geq$ 95 are indicated by filled purple circles. The color-coded group classifications (SPACA4, Bouncer, Lypc, LY6G6e/d, Spaca4l, CD59, Ly-6) follow the human, mouse or zebrafish protein nomenclature.

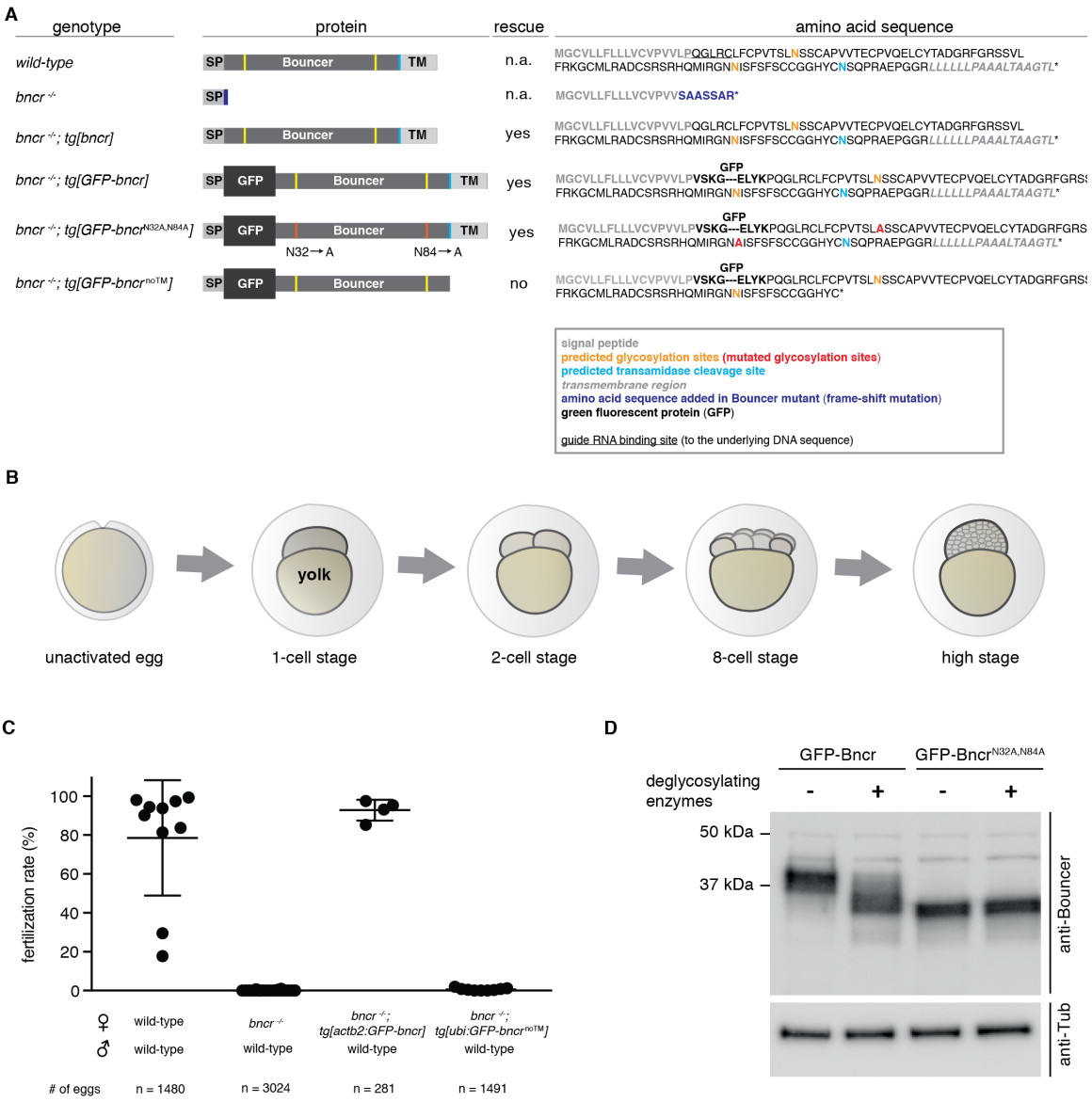

figure S3: Rescue ability of Bouncer protein variants

A – Schematic representation of Bouncer protein variants and their rescuing ability of *bncr*$^{-/-}$ females. The schemes indicate the signal peptide (SP), the two predicted N-glycosylation sites (yellow or red (mutated)), the transamidase cleavage site (turquoise) and the C-terminal transmembrane region (TM). Annotated full-length amino acid sequences are shown on the right. Note that for GFP, only the first and last four amino acids are shown. Wild-type: endogenous Bouncer; *bncr*$^{-/-}$: loss-of-function *bouncer* mutant (13-nt deletion after the signal peptide sequence); *tg[bncr]*: transgenically expressed Bouncer; *tg[GFP-bncr]*: transgenically expressed GFP-tagged Bouncer; *tg[GFP-bncr*^{N32A,N84A}*]*: transgenically expressed GFP-tagged non-glycosylatable Bouncer; *tg[GFP-bncr*^{noTM}*]*: transgenically expressed GFP-tagged Bouncer lacking the GPI-anchoring site and the C-terminal transmembrane region.

B – Scheme of the early development of zebrafish embryos.

C – Quantification of the fertilization rates of the indicated crosses. Mean ± SD are indicated. n = total number of eggs.
D – GFP-tagged Bouncer^{N32A,N84A} is not glycosylated. Treatment of GFP-Bncr^{N32A,N84A}-containing samples with deglycosylating enzymes does not affect the molecular size of GFP-Bncr^{N32A,N84A}, which is in contrast to treatment of GFP-Bncr-containing samples, which decreases in size. Twenty caps of dome stage embryos expressing GFP-Bncr or GFP-Bncr^{N32A,N84A} were loaded per lane. Bouncer was detected with an anti-Bouncer-specific antibody. Tubulin is shown as loading control.

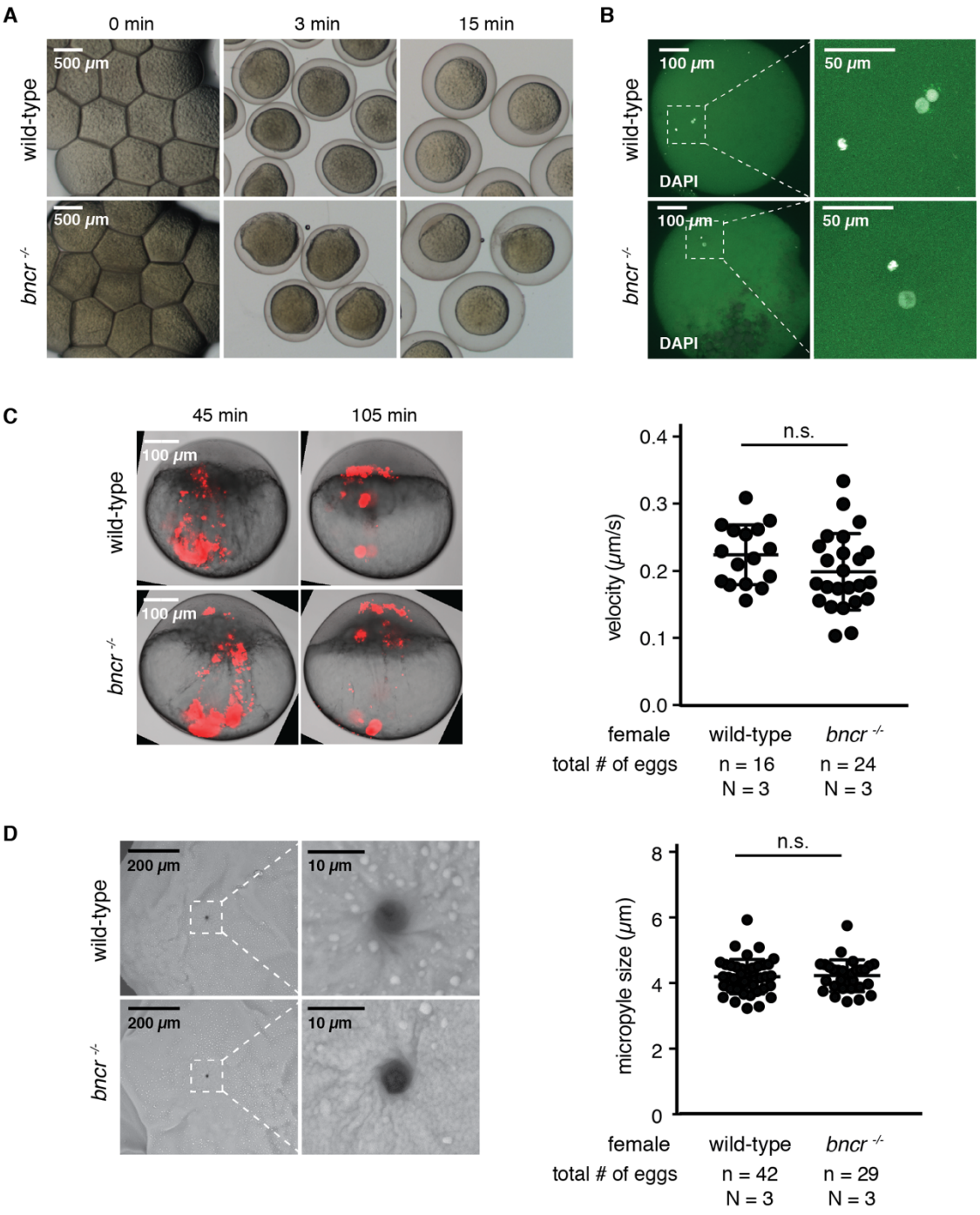

figure S4: Bouncer is not required for egg activation

A – *Bncr*$^{-/-}$ females produce morphologically normal eggs with normally elevated chorions similar to wild-type eggs after egg activation.

B – The polar body is extruded normally in Bouncer-deficient eggs. Representative image of fixed and DAPI-stained wild-type and Bouncer-depleted eggs ~15 minutes after mating. The wild-type egg contains three DAPI-stained spots (female nucleus, male nucleus, and more condensed polar body at the bottom left corner), while the Bouncer-depleted egg only contains two DAPI-stained spots (the female nucleus and more condensed polar body).

C – Cytoplasmic streaming is normal in *bncr*$^{-/-}$ eggs. Fluorescent beads were injected into wild-type or Bouncer-deficient eggs and bead movement was tracked. Left: Representative images. Right: Quantification of bead velocity. Each data point represents the average velocity of all tracked beads in one egg. Mean ± SD are indicated ($p = 0.141$, unpaired t-test; n = number of eggs; N = number of biological replicates).

D – The micropyle is present and of normal size in *bncr*$^{-/-}$ eggs. Left: Representative scanning electron microscopy images of unfertilized wild-type and *bncr*$^{-/-}$ eggs 30 seconds after activation, showing the presence of the micropyle in Bouncer-deficient eggs. Right: Quantification of the size of the micropyle based on analysis of Coomassie-stained chorions. Mean ± SD are indicated ($p = 0.804$, unpaired t-test; n = number of eggs; N = number of biological replicates).

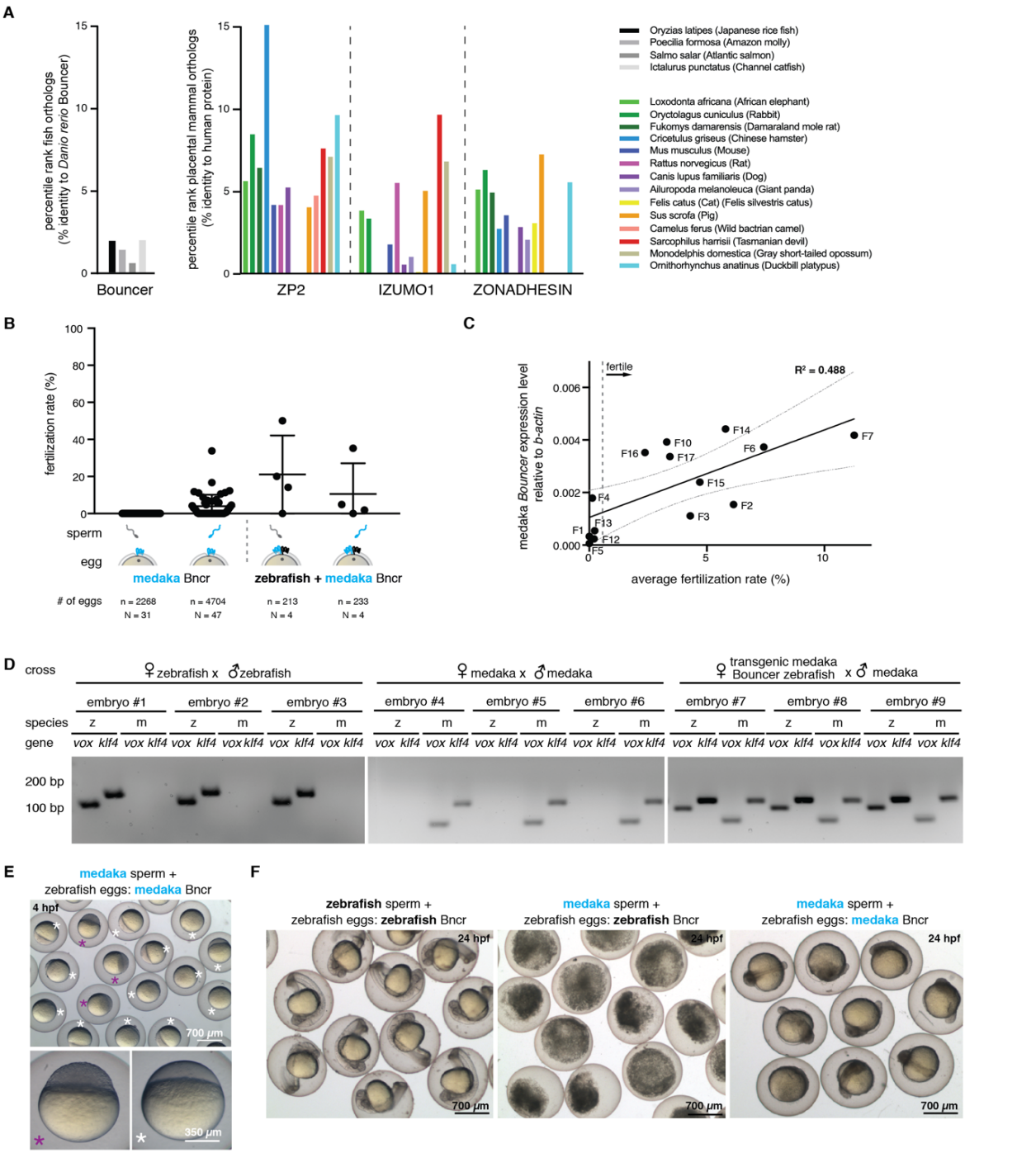

figure S5: Zebrafish eggs expressing medaka Bouncer can be fertilized by medaka sperm and develop into hybrid embryos

A – Fish Bouncers show similarly high levels of amino acid sequence divergence as known mammalian fertilization factors that have been implicated in species-specificity of fertilization. Plotted are percentile ranks of amino acid sequence identities between high-scoring pairs of fish Bouncers compared to zebrafish Bouncer (left), and placental mammal proteins compared to human fertilization factors (right). Species for which no orthologs could be identified with our

settings were left blank (see Materials and Methods for details). For example, a percentile rank of 10% means that 90% of pairwise orthologs between these two species have higher amino acid sequence identity values.

B - Medaka Bouncer is sufficient to allow entry of medaka sperm into zebrafish eggs. Left: while zebrafish sperm was unable to enter zebrafish *bncr*$^{-/-}$ eggs expressing medaka Bouncer, *in vitro* fertilization with medaka sperm resulted in an average fertilization rate of 3.7%. Data is shown for all 15 medaka Bouncer-expressing females tested. Right: zebrafish eggs expressing both zebrafish and medaka Bouncer can be fertilized by both zebrafish and medaka sperm. Mean ± SD are indicated. n = number of eggs; N = number of biological replicates (independent crosses).

C – Fertilization rates of individual medaka Bouncer zebrafish females correlate with the expression levels of medaka *bouncer* mRNA in eggs of the individual females (R^2 = 0.488). Plotted are average fertilization rates of individual medaka Bouncer zebrafish versus expression levels of medaka *bouncer* mRNA, normalized by *beta-actin* mRNA (expression values were measured in biological triplicates per female). One infertile female died before analysis and was not included. The curved dashed lines represent 95% confidence intervals for the line of best fit (linear regression analysis). Females were considered fertile if they exhibited an average fertilization rate > 0.5%.

D – Fertilization of zebrafish eggs expressing only medaka Bouncer by medaka sperm results in hybrid embryos. Both zebrafish and medaka genomic DNAs (gDNAs) are present in hybrid embryos. gDNAs of single zebrafish embryos (#1-3), medaka embryos (#4-6), and hybrid embryos (#7-9) were isolated at sphere stage (zebrafish and hybrid) or between stages 11-12 (medaka) and used for PCR. Zebrafish- and medaka-specific primers (indicated as “z” or “m”) were used to amplify *vox*- and *klf4*-specific gene regions.

E-F - Fertilization of zebrafish eggs expressing only medaka Bouncer yields zebrafish-medaka hybrid embryos that undergo gastrulation movements (E) and axis formation (F). Overview images were taken at 4 hpf (E) and 1 dpf (F). In (E), white asterisks indicate unfertilized embryos (resembling one-cell stage embryos), and purple asterisks indicate fertilized embryos.

table S1
Table with NCBI protein and cDNA IDs, sorted according to the phylogenetic tree (192 entries). Columns contain the following information: group (SPACA4, Bouncer, Lypc, LY6G6d/e, Spaca4l, CD59, Ly-6); ID in figures (in protein sequence alignment and phylogenetic tree); species; NCBI protein accession number (if available); NCBI nucleotide (mRNA or genomic) accession number (if available); description (retrieved from the NCBI protein entry); comment on coding sequence retrieval. *Oryzias latipes* sequences were retrieved by conceptual translation, and the sequence of *Squalus acanthias* was retrieved by contig assembly and conceptual translation.

movie S1
Cytoplasmic streaming is normal in Bouncer-depleted eggs. Shown are representative time-lapse series for a wild-type and *bncr*$^{-/-}$ embryo that were injected with FluoroSpheres immediately after egg activation.

movie S2
Sperm can approach the micropyle area in Bouncer-depleted eggs. Shown are representative time-lapse series for MitoTracker-labelled wild-type sperm (magenta) approaching the micropyle area of a wild-type and a *bncr*$^{-/-}$ egg. Imaging started immediately after sperm addition.

data S1
Coding sequences of members of the Ly6/uPAR family (192 entries)

data S2
Protein sequences of members of the Ly6/uPAR family (192 entries)