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

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Abstract

Fertilization is fundamental for sexual reproduction, yet its molecular mechanisms are poorly understood. We found that an oocyte-expressed Ly6/uPAR protein, which we call Bouncer, is a crucial fertilization factor in zebrafish. Membrane-bound Bouncer mediates sperm-egg binding and is thus essential for sperm entry into the egg. Remarkably, Bouncer not only is required for sperm-egg interaction but is also sufficient to allow cross-species fertilization between zebrafish and medaka, two fish species that diverged more than 200 million years ago. Our study thus identifies Bouncer as a key determinant of species-specific fertilization in fish. Bouncer's closest homolog in tetrapods, SPACA4, is restricted to the male germline in internally fertilizing vertebrates, which suggests that our findings in fish have relevance to human biology.

Keywords

fertilization; species-specificity; sperm-egg interaction; zebrafish

Introduction, results and discussion

Fertilization, whereby two gametes fuse to form the single-cell zygote in sexually reproducing organisms, is highly efficient, yet species-restricted. This strategy ensures reproductive success and the survival of distinct species. However, the means by which nature has fulfilled these seemingly contradictory requirements, particularly at the molecular level, have remained a mystery. The only vertebrate proteins known so far to be essential for

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Author contributions

S.H. and A.P. conceived the study; S.H. performed most experiments except experiments regarding species-specificity, which were performed by K.R.G., and generation of RNA-seq data and *bncr*^{-/-} fish, which were performed by A.P.; S.H., K.R.G., and A.P. analyzed the data. A.S. and A.P. performed the phylogenetic analysis; and S.H. and A.P. wrote the manuscript with input from K.R.G. and A.S.

Competing interests

The authors declare no competing interests.

Data and materials availability

RNA-seq data first reported here were deposited at the Gene Expression Omnibus (GEO) and are available under GEO accession number GSE111882. All other data are available in the manuscript or the supplementary material.

sperm-egg binding are the sperm-expressed IZUMO1 (1, 2) and the egg membrane proteins JUNO (3) and CD9 (4–6). Binding of IZUMO1 to JUNO mediates adhesion between sperm and egg in mammals (1–3, 7), whereas the role of CD9 in this process remains unclear. Although *in vitro* binding assays show that human IZUMO1 binds more efficiently to human JUNO than to mouse JUNO (8), an *in vivo* function in mediating species-specificity has not been identified for any of these factors.

To identify factors required for fertilization in vertebrates, we examined our collection of predicted protein-coding genes (9) that are expressed in zebrafish oocytes and/or testis. A single-exon gene stood out because of its high expression in zebrafish oocytes (Fig. 1A) and the presence of homologous sequences in other vertebrates. On the basis of its loss-of-function phenotype (see below), we named this gene *bouncer* (*bncr*) in reference to the colloquial name of a security guard at a bar. Although *bouncer* lacks any gene annotation in the newest zebrafish genome release (GRCz11), our RNA sequencing (RNA-seq) and *in situ* hybridization analyses (fig. S1A, B), ribosome profiling data (9–11), and cap analysis gene expression (CAGE)-based transcription start site analysis (12) suggested that *bouncer* is a maternal transcript that generates a mature 80-amino acid glycosylphosphatidylinositol (GPI)-anchored protein (Fig. 1A). Consistent with two predicted N-glycosylation sites (Fig. 1A), a Bouncer-specific antibody detected glycosylated Bouncer in the egg (Fig. 1B).

A protein domain search classified Bouncer as a member of the Ly6/uPAR (Ly6/urokinase-type plasminogen activator receptor) protein superfamily, which includes proteins as diverse as toxins, immunoregulators, and cell surface receptors (13). This protein family is characterized by a 60- to 80-amino acid domain containing 8 to 10 highly conserved cysteines that form a three-finger structure (Fig. 1A, C; fig. S2A). Apart from the cysteines, other amino acids have diverged substantially within this protein superfamily (Fig. 1C; fig. S2A and B). BLASTP searches with zebrafish Bouncer and phylogenetic sequence analyses suggested that SPACA4 is the closest homolog in mammals, reptiles, and amphibians (Fig. 1C; fig. S2A-C; data S1 and 2 and table S1). Human SPACA4/SAMP14 (sperm acrosome membrane-associated protein 4/sperm acrosomal membrane protein 14) was originally identified in a proteomics study as a sperm acrosomal protein, and *in vitro* experiments implied a possible function in fertilization (14). However, the *in vivo* function and importance of SPACA4 are unknown.

Intrigued by our finding that zebrafish Bouncer is expressed in oocytes and that its closest homolog in humans was reported to be expressed in sperm (14), we analyzed the expression patterns of other Bouncer/SPACA4 homologs. We found that externally fertilizing vertebrates (e.g. fish and amphibians) show oocyte-restricted expression, whereas all internally fertilizing vertebrates analyzed (e.g. reptiles and mammals) show testis-specific expression (Fig. 1C; Fig. S2A). Together, these results identified Bouncer and SPACA4 as homologs with opposing sex-specific, germline-restricted expression patterns in externally versus internally fertilizing vertebrates.

To investigate the function of Bouncer, we used CRISPR/Cas9 to generate *bouncer* knockout zebrafish. We established a stable mutant line with a *bouncer* allele carrying a 13-nucleotide deletion, which abolishes the production of mature Bouncer protein (Fig. 1B; fig. S3A).

Incrosses of *bouncer* heterozygous (*bncr*^{+/-}) fish gave rise to homozygous mutant adults (*bncr*^{-/-}) at a Mendelian ratio of ~25%, which suggests that Bouncer is not essential for development. However, *in vivo* mating experiments showed that only 7 of 3024 eggs (0.11%) derived from *bncr*^{-/-} females developed into cleavage-stage embryos, as opposed to the majority of eggs from wild-type or *bncr*^{+/-} females or from wild-type females fertilized by *bncr*^{-/-} males (Fig. 1D, E; fig. S3A and B). Notably, female near-sterility was fully rescued by ubiquitous expression of transgenic untagged or green fluorescent protein (GFP)-tagged Bouncer (Fig. 1D, E; fig. S3A and C), which confirms that the observed defect was indeed due to the lack of Bouncer protein. Ubiquitous expression of a Bouncer mutant that cannot be glycosylated (GFP-Bncr^{N32A,N84A}; fig. S3A and D) also fully rescued female near-sterility (Fig. 1E); this finding demonstrates that glycosylation of Bouncer does not contribute to its function. Thus, oocyte-expressed Bouncer protein is necessary for efficient reproduction in zebrafish.

Zebrafish eggs are activated upon contact with spawning medium, independently of the presence of sperm. Egg activation appeared unaffected in eggs from *bncr*^{-/-} females, as was evident by normal elevation of the chorion (the outer protective envelope of fish embryos), polar body extrusion, and cytoplasmic streaming (fig. S4A-C; movie S1). Moreover, the micropyle, an opening in the chorion that serves as the sole entry point for sperm into zebrafish eggs, is present in *bncr*^{-/-} eggs, and its size is similar to that of wild-type eggs (fig. S4D). These results suggest that Bouncer is not required for egg activation and micropyle formation.

Because eggs from *bncr*^{-/-} females lack any apparent morphological defects, yet do not develop beyond the one-cell stage, Bouncer might be required for fertilization and/or the initiation of early cleavage cycles. To distinguish between these possibilities, we first asked whether sperm can enter eggs lacking Bouncer. *In vitro* fertilization (IVF) of wild-type and Bouncer-deficient eggs with MitoTracker-labeled sperm allowed us to detect sperm only in wild-type eggs (50%), but never in Bouncer-deficient eggs (Fig. 2A), which suggests that Bouncer might play a role in sperm entry during fertilization. Consistent with the idea that Bouncer's sole function is to allow sperm to enter the egg, delivery of sperm into Bouncer-deficient eggs by intra-cytoplasmic sperm injection (ICSI) bypassed the requirement for Bouncer and restored embryonic development beyond the one-cell stage (Fig. 2B and fig. S3B). Bouncer's key function is therefore in enabling sperm entry during fertilization.

To gain further insight into Bouncer's function, we assessed its localization. Consistent with its predicted GPI-anchorage, confocal imaging revealed that the fully functional, GFP-tagged Bouncer (Fig. 1E) localized to the egg membrane and to vesicles within the egg (Fig. 3A). Further, ubiquitous expression of a version of Bouncer lacking the C-terminal membrane anchor (GFP-Bncr^{noTM}) did not rescue the near-sterility of *bncr*^{-/-} females (fig. S3A and C), which suggests that membrane localization of Bouncer is required for its function.

The requirement for Bouncer at the egg membrane implies that it could promote the approach of sperm to the egg or sperm-egg binding/fusion. Live cell imaging revealed that multiple MitoTracker-labeled sperm are recruited to the micropyle independently of Bouncer

(Fig. 3B; movie S2). Thus, Bouncer does not provide an essential attractive cue that guides sperm towards the egg/micropyle.

During live imaging, multiple sperm entered the narrow opening of the micropyle simultaneously, rendering a more detailed analysis of sperm-egg binding capability infeasible. To investigate Bouncer's potential role in sperm-egg binding, we exposed the entire egg surface to sperm by removing the chorion. MitoTracker-labeled sperm remained bound to the surface of wild-type eggs in large clusters (>10 sperm) (Fig. 3C and D). In contrast, only a few individual sperm (<10) remained attached to the majority of Bouncer-deficient eggs ($P < 0.002$) (Fig. 3C and D). These results suggest that Bouncer promotes sperm-egg binding.

Bouncer shows a high degree of amino acid sequence divergence among different fish species, similar to other proteins involved in species-specificity in mammals (fig. S5A). This raised the interesting possibility that Bouncer might contribute to the species-specificity of fertilization in fish. To test this hypothesis, we generated *bncr*^{-/-} zebrafish that ubiquitously express medaka Bouncer (*bncr*^{-/-}; tg[*ubi:medaka-bncr*]), henceforth called transgenic medaka Bouncer fish. Medaka was chosen because of its large evolutionary distance from zebrafish (~200 million years), its inability to cross-hybridize with zebrafish, and the low (40%) amino acid identity between the mature zebrafish and medaka Bouncer proteins (Fig. 4A). Expression of medaka Bouncer in *bncr*^{-/-} females did not efficiently rescue fertility when crossed to wild-type male zebrafish (average fertilization rate of 0.45%, versus 0.11% for *bncr*^{-/-} and 78.6% for wild-type females) (Fig. 4B), supporting our hypothesis that Bouncer might influence species-specific gamete interaction. To directly test this possibility, we performed a series of IVF experiments. As expected, wild-type zebrafish eggs exhibited high fertilization rates with zebrafish sperm (average rate of 48.3%) but were not fertilized by medaka sperm (Fig. 4C). Moreover, zebrafish *bncr*^{-/-} eggs were fertilized by neither sperm (Fig. 4C). Remarkably, eggs from transgenic medaka Bouncer females were fertilized by medaka, but not zebrafish, sperm (Fig. 4C), and eggs from females expressing both medaka Bouncer and zebrafish Bouncer could be fertilized by both sperm (fig. S5B). The average fertilization rate of all transgenic medaka Bouncer females (15) tested was 3.9% (fig. S5B). Whereas 6 of 15 females were infertile (average fertilization rate < 0.5%; fig. S5C), eggs from the remaining nine females were fertilized by medaka sperm at an average rate of 5.7% (Fig. 4C). Fertility rates of individual transgenic medaka Bouncer females were found to correlate with expression levels of medaka *bouncer* mRNA in eggs (fig. S5C), supporting a causal link between medaka Bouncer expression and medaka sperm entry. The resulting embryos were zebrafish-medaka hybrids and not haploid zebrafish embryos (fig. S5D). Hybrid embryos underwent cell cleavage and gastrulation (fig. S5E) and displayed anterior-posterior axis formation after 24 hours (Fig 4D; fig. S5F) but did not survive past 48 hours. These results demonstrate that Bouncer is necessary and sufficient for mediating species-specific fertilization in fish. Like a security guard, Bouncer is decisive for allowing conspecific sperm to enter, while keeping heterospecific sperm out. This is the first report of a protein in any organism allowing entry of another species' sperm.

Our finding that ectopic expression of another species' Bouncer is sufficient to allow cross-species fertilization strongly suggests that Bouncer has a direct, species-specific interaction

partner on sperm. Additionally, the low average fertilization rate (5.7%) of medaka Bouncer-expressing zebrafish eggs by medaka sperm implies that other factors likely contribute to species-specific sperm-egg interaction. The identification of these factors and Bouncer's interaction partner on sperm will be crucial to unraveling the mechanism of species specificity of fertilization.

Thus far, the only known interacting membrane-bound proteins on vertebrate sperm and egg are IZUMO1 and JUNO in mammals (1–3, 7). Whether these two proteins also play a role in mediating species-specific fertilization *in vivo* is, however, still unclear (8). In many organisms, species-specificity of fertilization is mediated between proteins on the sperm membrane and those localized to the egg coat (15, 16). For example, in sea urchin, the vitelline envelope protein EBR1 (egg bindin receptor protein-1) binds specifically to the sperm membrane protein bindin (17–19). Similarly, the egg coat protein VERL of abalone is species-specifically bound by lysin, a small secreted protein from sperm (20–23). Whereas lysin has no known homolog in vertebrates, VERL shows structural homology to the mammalian zona pellucida protein ZP2 (22), which was shown to be involved in species-specific binding of sperm to the zona pellucida in mouse and humans (24). Furthermore, from the side of the sperm, the mammalian sperm acrosomal protein zonadhesin binds species-specifically to the zona pellucida, even though it is not required for fertility (25). In contrast to these proteins, Bouncer mediates species-specific binding of sperm to the egg membrane, not to the egg coat.

Bouncer and SPACA4, both members of the large Ly6/uPAR superfamily, have opposing germline-specific expression patterns in externally versus internally fertilizing organisms. The underlying reason for this is unclear, but one can speculate that in externally fertilizing species, oocyte expression of Bouncer contributes to post-copulatory female mate choice (also called cryptic female mate choice) (26). Vertebrates performing external fertilization cannot guarantee that only conspecific sperm reaches the egg by pre-copulatory mate choice (27, 28). Oocyte-expressed proteins such as Bouncer could therefore support the selection of conspecific sperm. Our work on Bouncer also raises the intriguing possibility that SPACA4 might play an important role in mammalian fertilization, albeit from the side of the male. Although a knockout for murine *Spaca4* has not yet been reported, this idea is consistent with the localization of SPACA4 to the inner acrosomal membrane of sperm and the observed reduction of sperm-egg binding and fusion *in vitro* by incubation of sperm with antibody to SPACA4 (14). Future experiments that address the *in vivo* function of mammalian SPACA4 during fertilization will therefore be of interest. Given that both genes are restricted to the germline, our findings in fish may have direct relevance for fertilization in mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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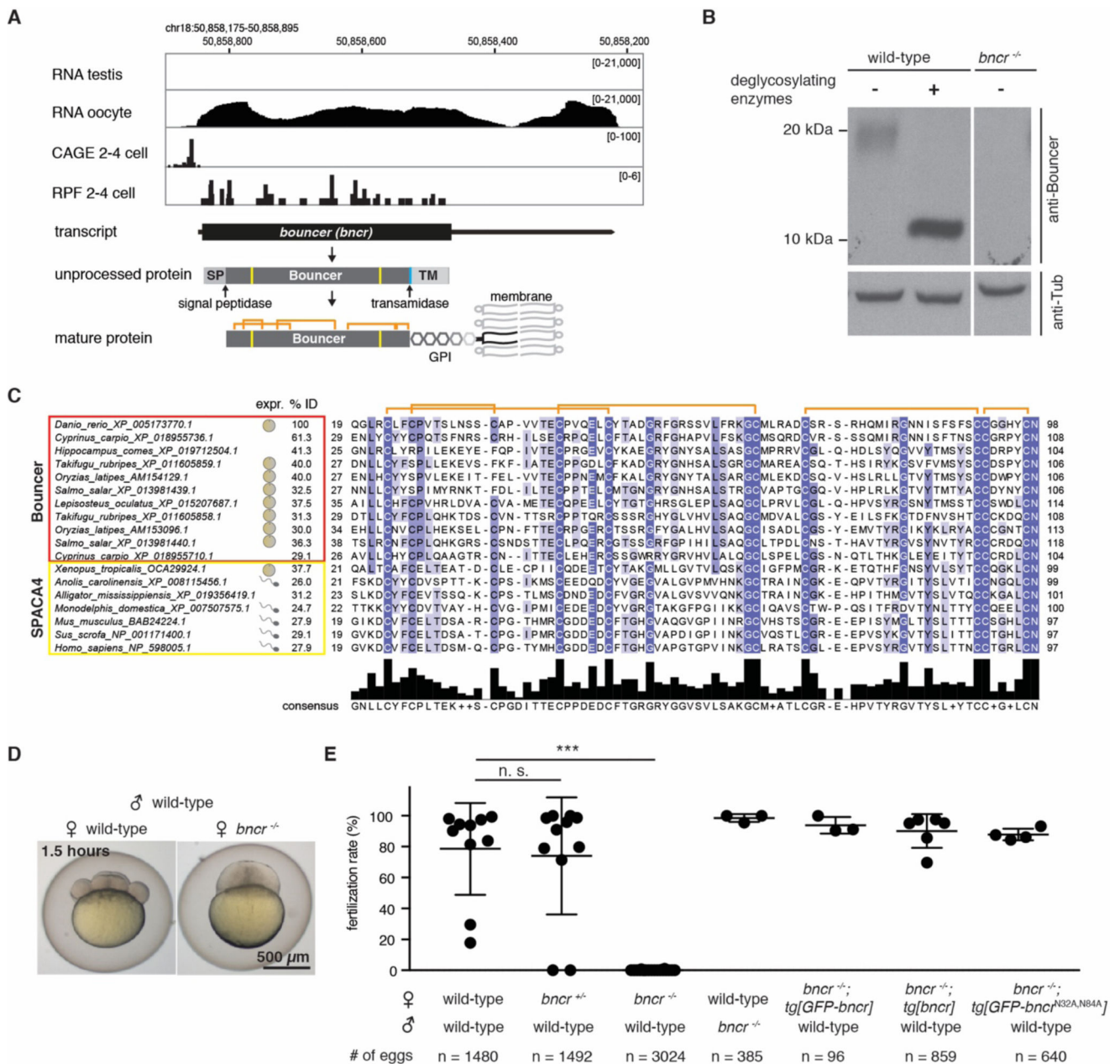


Figure 1. Identification of Bouncer in fish.

(A) Expression and genomic features of Bouncer. Coverage tracks for RNA sequencing, ribosome profiling (RPF) (10), and CAGE data (12) are shown. Genomic coordinates are based on GRCz10. SP, signal peptide; TM, transmembrane region; orange, predicted disulfide bonds; yellow, predicted N-glycosylation sites; turquoise, predicted transamidase cleavage site. (B) Endogenous Bouncer protein is glycosylated. Endogenous Bouncer is detected in the zebrafish egg by a Bouncer-specific antibody at a higher molecular weight than predicted (~20 kDa) but shifts down to the expected size (10 kDa) after treatment with deglycosylating enzymes. No Bouncer signal is detected in eggs from *bncr*^{-/-} mutant females. (C) Protein sequence alignment of the mature domain of Bouncer/SPACA4 protein

family members. Apart from the well conserved cysteines (orange denotes predicted disulfide bonds), Bouncer/SPACA4 shows high amino acid divergence among different species (% ID, percent sequence identity to the mature domain of zebrafish Bouncer). The extent of the mature domain displayed here is based on the prediction for zebrafish Bouncer. For all species for which expression data were available [(29–33): human expression based on GTEx Portal; expressed sequence tags based on NCBI], *bouncer/Spaca4* RNA is restricted to either the male (symbol: sperm) or female (symbol: egg) germline. For sequences and accessions see data S1 and S2 and table S1. Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. **(D and E)** Lack of Bouncer causes near-sterility in female zebrafish. **(D)** Representative images of a developing, eight-cell stage embryo derived from a wild-type female, and an arrested, one-cell stage egg derived from a *bncr*^{-/-} female 1.5 hours after mating. **(E)** Transgenically expressed, *ubiquitin* promoter driven untagged, GFP-tagged, and non-glycosylatable Bouncer rescue the mutant phenotype. Data are means \pm SD; n = number of eggs. ***P < 0.0001 (Kruskal-Wallis test with Dunn multiple comparisons test); n.s., not significant.

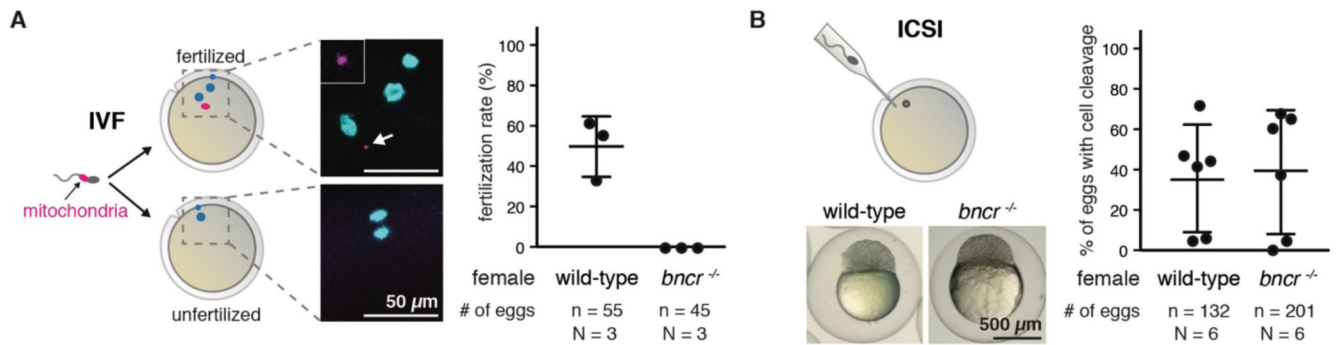


Figure 2. Bouncer is required for sperm entry into the egg.

(A) Sperm does not enter *bncr*^{-/-} eggs. Left: Experimental setup. Wild-type sperm was stained with MitoTracker label and used for IVF of wild-type and *bncr*^{-/-} eggs. Representative images are shown (arrow: MitoTracker signal, enlarged in white box). Right: Percentage of fertilized eggs, as indicated by the presence of one MitoTracker-labeled sperm, enlarged three DAPI signals (male nucleus, female nucleus, polar body). Means \pm SD are indicated. (B) ICSI is able to rescue *bncr*^{-/-} eggs. Top left: Experimental setup. Wild-type sperm was injected into wild-type or *bncr*^{-/-} eggs. Cell cleavage was scored after 3 hours. Bottom left: Representative images. Right: Percentage of eggs that show cell cleavage. Means \pm SD are indicated. n = total number of eggs; N = number of biological replicates.

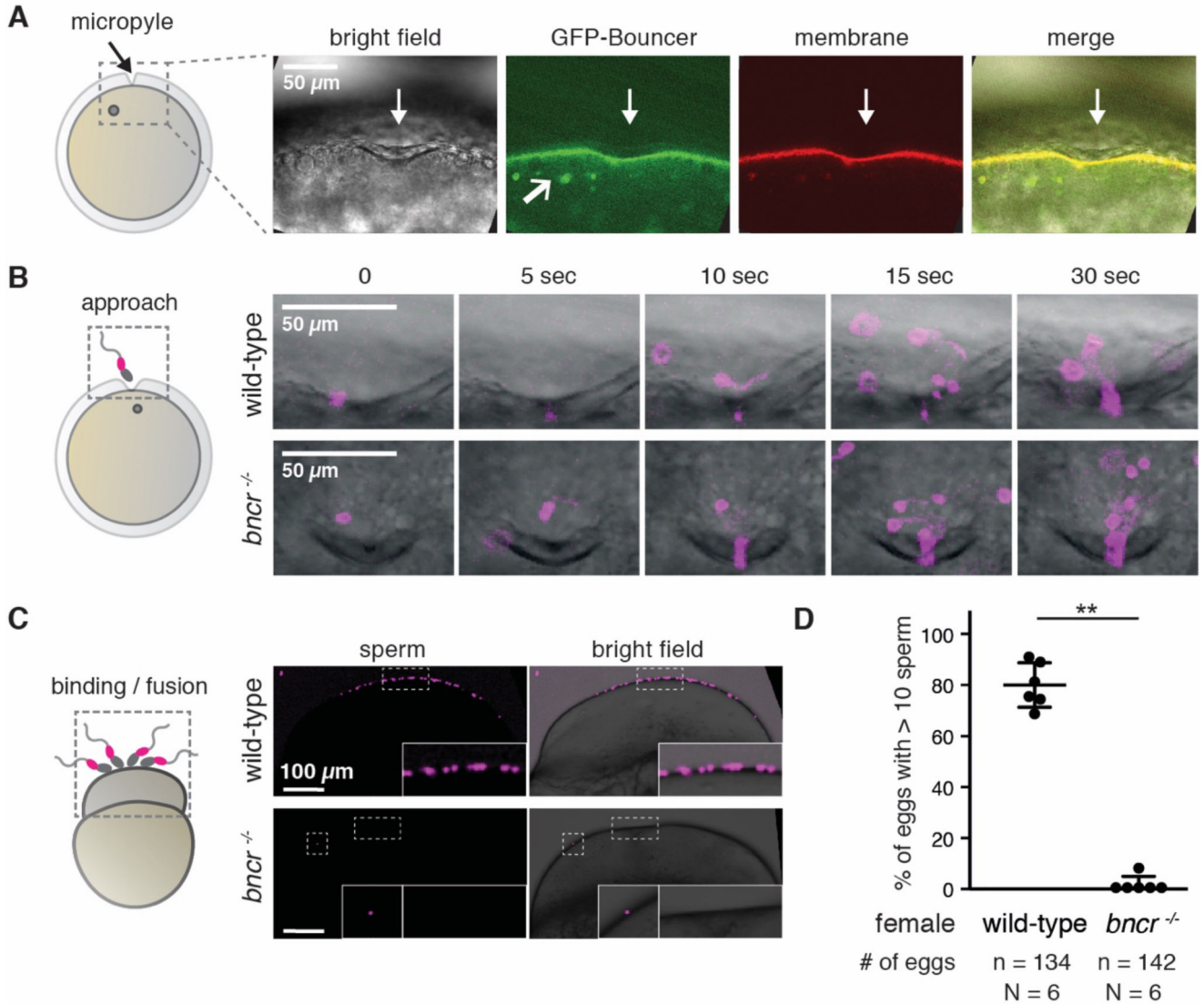


Figure 3. Bouncer mediates binding between sperm and egg.

(A) Bouncer localizes to the egg membrane and to vesicles. Confocal images of eggs expressing GFP-tagged Bouncer (green) and lyn-Tomato (membrane, red) during egg activation show that Bouncer localizes to the egg membrane around the micropyle (downwards pointing white arrow) and to vesicles (angled white arrow in GFP-Bouncer panel). Gray circle, egg nucleus. (B) Bouncer is not required for sperm approach. Left: Experimental setup. Right: Representative time series of multiple MitoTracker-labeled wild-type sperm (magenta) approaching the micropyle area of wild-type (top) and *bncr*^{-/-} (bottom) eggs. (C) *bncr*^{-/-} eggs are impaired in sperm-egg binding. Left: Experimental setup. Activated and dechorionated wild-type and *bncr*^{-/-} eggs were incubated with MitoTracker-labeled wild-type sperm and gently washed. Right: Representative images of a wild-type egg (top, scored as >10) and a *bncr*^{-/-} egg (bottom) with a single bound sperm. Boxed areas are also shown at higher magnification. (D) Quantification of sperm-egg binding. Eggs were

classified as either >10 sperm bound or <10 sperm bound. Data are means \pm SD ($P < 0.002$, Mann-Whitney test; n = number of eggs; N = number of biological replicates).

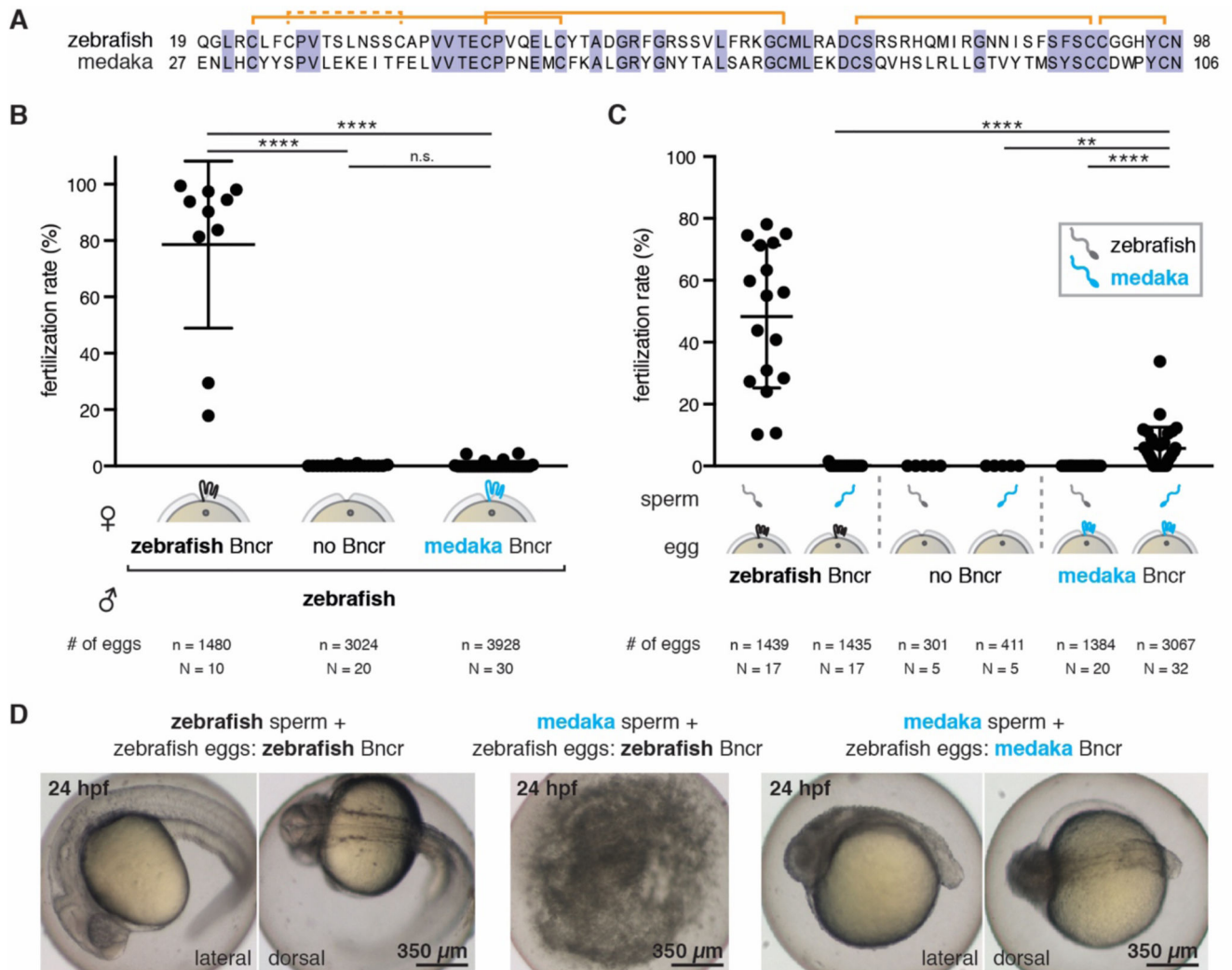


Figure 4. Bouncer mediates species-specific fertilization.

(A) Mature zebrafish and medaka Bouncer have only 40% amino acid sequence identity. Orange denotes predicted disulfide bonds; the dashed orange line denotes a disulfide bond predicted in zebrafish but not in medaka. (B) Medaka Bouncer does not efficiently rescue the fertilization defect of zebrafish *bncr*^{-/-} females. Means ± SD are indicated [Kruskal-Wallis test with Dunn multiple-comparisons test: wild-type (wt) x wt versus *bncr*^{-/-} x wt, adj. P**** < 0.0001; wt x wt versus medaka Bouncer x wt, adj. P**** < 0.0001; *bncr*^{-/-} x wt versus medaka Bouncer x wt, n.s.; n = number of eggs; N = number of biological replicates]. (C) Medaka Bouncer is sufficient to allow entry of medaka sperm into zebrafish eggs. Medaka sperm did not fertilize wild-type zebrafish eggs, but medaka Bouncer expressing zebrafish eggs had an average fertilization rate of 5.7% in IVF experiments. Data are shown for the subset of medaka Bouncer expressing females (9 of 15) that were fertile (see fig. S5B for data of all 15 females tested). Data are means ± SD (Kruskal-Wallis test with Dunn multiple-comparisons test: medaka sperm on zebrafish versus medaka Bouncer eggs, adj. P**** < 0.0001; zebrafish sperm on medaka Bouncer eggs versus medaka sperm on medaka

Bouncer eggs, adj. $P^{****} < 0.0001$; medaka sperm on *bncr*^{-/-} zebrafish eggs versus medaka Bouncer eggs, adj. $P^{**} = 0.0052$; n = number of eggs; N = number of biological replicates). **(D)** Fertilization of zebrafish eggs expressing only medaka Bouncer yields medaka-zebrafish hybrid embryos. Left: Wild-type zebrafish embryos fertilized by zebrafish sperm. Center: Wild-type zebrafish embryos are not fertilized by medaka sperm and decompose within 24 hours. Right: Zebrafish eggs expressing only medaka Bouncer are fertilized by medaka sperm and develop into hybrid embryos. hpf, hours post-fertilization.