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NELL2-mediated lumicrine signaling through OVCH2 is required for male fertility

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Abstract

The lumicrine system is a postulated signaling system in which testis-derived (upstream) secreted factors enter the male reproductive tract to regulate epididymal (downstream) pathways required for sperm maturation. Until now, no lumicrine factors have been identified. We demonstrate that a testicular germ-cell secreted EGF-like protein, NELL2, specifically binds to an orphan receptor tyrosine kinase, ROS1, and mediates the differentiation of the initial segment (IS) of the caput epididymis. *Nell2* knockout male mice are infertile. We further show that the IS-specific secreted

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Supplementary Materials:
Materials and Methods
Figures S1–S9
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proteases, OVCH2 and ADAM28, are expressed upon IS maturation, and OVCH2 is required for processing of the sperm surface protein, ADAM3, which is required for sperm fertilizing ability. Thus, we have uncovered a lumicrine system essential for testis-epididymis-spermatozoa (NELL2-ROS1-OVCH2-ADAM3) signaling and male fertility.

One Sentence Summary:

Testicular NELL2 induces ROS1-mediated epididymal maturation and secretion of OVCH2 to ensure sperm function.

Sex hormones, especially androgens, play critical roles in male genital tract development and function. In addition to androgens, it is hypothesized that proteins and/or small molecules produced in the mammalian testis (upstream) are secreted into the luminal space and act downstream on the epididymis to regulate, maintain, and/or differentiate the epididymis (downstream) (1). However, the molecular entities of this lumicrine system have until now not been identified. Interestingly, an orphan receptor tyrosine kinase ROS1, which is well known as oncogenic when its gene is rearranged to form active fusion proteins, is expressed in the initial segment (IS) of the caput epididymis and indispensable for postnatal IS differentiation (2). Neither the testicular factors that regulate IS differentiation nor the mechanisms by which differentiated IS matures spermatozoa has been fully clarified. In this study, we identified a testis-secreted factor that binds to ROS1 and is indispensable for IS differentiation and male fertility.

The dependency of IS on testicular lumicrine factors is reported to be established between postnatal-day (P) 15 to P19 (3). We found that germ cell-deficient *Kit*$^{W/Wv}$ (*W*/*W*^{v}) mice also show impaired IS differentiation (fig. S1). These findings strongly suggest that the putative ROS1 ligand is expressed by developing testicular germ cells. To identify the testicular ligand for epididymal orphan receptor ROS1, we screened for testis-secreted proteins that are upregulated during spermatogenesis (fig. S2). First, from GSE640 Microarray data (36,939 spots), we selected 1,706 spots as matrisome genes *in silico* (4). Second, we chose nine candidate proteins satisfied the criteria that we set (i.e., proteins with N-terminal signal sequence, no transmembrane domain, and average transcript levels more than three times higher after P18 compared with those before P14). We excluded seven genes in which knockout (KO) mice lacking these proteins were fertile (i.e., *Comp*, *Vit*, *Zp3r*, *C1qtnf4*, and *Agt*) (5–9) or showed different phenotypes from *Ros1* KO mice (i.e., *Insl6* and *Prss21*)(10, 11). Because PRSS39 is a protease and less likely to be a ROS1 ligand, we focused on neural EGFL-like 2 (NELL2), which satisfied the criteria above and could be a hypothetical lumicrine factor and regulator of male fertility.

When examined *in vitro*, recombinant NELL2 protein specifically bound recombinant ROS1, whereas recombinant COMP, which carries EGF-like domains, did not (Fig. 1A and fig. S2). In WT mice, *Nell2* is expressed in testis and brain but not in epididymis (Fig. 1B and fig. S1). In *W*/*W*^{v} mice, *Nell2* is expressed in brain, but the IS does not differentiate (fig. S1). Therefore, we focused on testis as the source of NELL2 and identified spermatocytes as *Nell2*-expressing cells with single cell RNAseq data (fig. S1)(12), consistent with increased *Nell2* transcript levels from P14 (fig. S2). As spermatocytes are located inside blood-testis

barrier in seminiferous tubules, these data strongly suggest that NELL2 secreted from testicular germ cells can transit through the luminal space of seminiferous tubules and bind to ROS1 on the epididymal epithelial cells.

To investigate NELL2 functions *in vivo*, we generated *Nell2* KO mice using CRISPR/Cas9 (fig. S3) and found that *Nell2* KO males are sterile (Fig. 1C). A *Clgn-Nell2* transgene, which expresses *Nell2* in a testicular germ cell-specific manner (13), completely rescued the male infertility of the *Nell2* KO mice, again excluding a possibility that NELL2 expressed in the brain regulates fertility (fig. S4). Further, we succeeded in detecting testis-secreted His-tagged NELL2 in the luminal contents of the epididymal duct, supporting that NELL2 is a lumicrine factor (fig. S4).

To determine the cause of infertility in *Nell2* KO males, we examined their reproductive tracts. Spermatogenesis appeared not affected in *Nell2* KO testis (fig. S3). In contrast, the IS was poorly differentiated in *Nell2* KO males (Fig. 1D and E; fig. S3 and S5) similar to *Ros1* KO mice (2). IS differentiation begins at 2–3 wks of age and corresponds with spatiotemporal *Nell2* expression in testis, and this postnatal differentiation of the IS was completely abolished in *Nell2* KO males and continued throughout life (fig. S5).

ERK1/2, a signal mediator whose phosphorylation depends on testicular lumicrine factors (14), is upregulated from 2–3 wks postnatally in parallel with IS differentiation (15). While the ERK1/2 signal is downregulated in *Ros1* mutant epididymis (15), it is hypo-phosphorylated in *Nell2* KO caput epididymis (Fig. 1F–H). Transcription factors *Etv1*, *Etv4*, and *Etv5*, which are located downstream of ERK signaling, are downregulated in *Nell2* KO IS (Fig. 1I). These results strongly suggest that the molecular basis of impaired IS differentiation is common between *Nell2* KO mice and *Ros1* KO mice.

We next characterized the relationship between impaired IS differentiation and male infertility. When spermatozoa ejaculated into female reproductive tract are visualized with an *Acr-egfp* transgene (16, 17), *Nell2* KO spermatozoa are observed in the uterus, but not in the oviduct (Fig. 2A–F), indicating that the *Nell2* KO spermatozoa are deficient in the passage through the utero-tubal junction (UTJ), as observed in *Ros1* KO mice (18). Further, both KO mice showed lack of sperm binding ability to zona pellucida (ZP), the oocyte extracellular matrix (Fig. 2G and H; fig. S6).

The inability of spermatozoa to pass through the UTJ and to bind to the ZP is often caused by loss of mature a disintegrin and metalopeptidase 3 (ADAM3), a spermatozoa surface transmembrane protein, in cauda epididymal mature spermatozoa (19). ADAM3 is expressed in testicular germ cells (TGCs) as a precursor N-linked glycosylated protein of ~100 kD, and it becomes processed by an unknown mechanism to a mature form of ~25 kD during sperm transit through the epididymis(20). We found that precursor ADAM3 is not processed correctly to mature ADAM3 in *Nell2* KO cauda epididymal spermatozoa (Fig. 2I and J). Expression of other fertility-related sperm proteins was not critically affected in *Nell2* KO testis and spermatozoa (fig. S3 and Fig. 2I). Transgenic rescue of *Nell2* KO males with testis-specific NELL2 expression restored sperm ADAM3 processing (fig. S4). These

results indicate that *Nell2* KO epididymis is missing a key protease involved in proper ADAM3 processing.

We hypothesized that a luminal environment suitable for the maturation of spermatozoa, including ADAM3 processing, is provided by fully differentiated IS but is impaired in *Nell2* KO epididymis. We examined the expression of genes encoding proteases in caput epididymis by RNAseq analysis (fig. S7 and Fig. 2K). By RNAseq and immunoblot analyses, we found that two proteases, OVCH2 (Ovochymase 2) and ADAM28, were strongly expressed in WT caput epididymis (fig. S7C) but highly downregulated in *Nell2* KO caput epididymis at the transcript and protein levels (Fig. 2K and L). As previously reported with ADAM28 (21), recombinant OVCH2 showed protease activity *in vitro* (fig. S7).

To determine if epididymal secreted proteases are necessary for ADAM3 processing and male fertility, we generated *Ovch2* KO and *Adam28* KO mice using CRISPR/Cas9 (Fig. 3, fig. S8, 9). KO mice lacking the epididymis-specific protease, OVCH2, partially reproduced the *Nell2* KO phenotype: while IS differentiation is not compromised in *Ovch2* KO mice (Fig. 3A and B), ADAM3 processing in the *Ovch2* KO spermatozoa is abnormal (Fig. 3C and D), and *Ovch2* KO male mice are sterile (Fig. 3E) due to inability of the sperm to transit the UTJ (Fig. 3F–K) and bind to the ZP (Fig 3L and M). In contrast, *Adam28* KO males were found to be fertile (fig. S9). Consistent with our model that OVCH2 is downstream of NELL2, *Ovch2* is not only epididymis-specific (fig. S7C), but OVCH2 protein is exquisitely localized to the IS of the caput epididymis (Fig. 4A), most proximal to the testis. These results indicate that an epididymal-specific and secreted protease, OVCH2, is required for sperm ADAM3 processing and consequential sperm fertilizing ability.

An intriguing mechanism proven in this study is the presence of a lumicrine signal pathway in which a testis-secreted protein (NELL2) signals through a ROS1-pathway to regulate the epididymal IS maturation and subsequent secretion of an epididymis protease (OVCH2) that processes ADAM3 for sperm fertilizing ability (Fig. 4B–D). NELL2 is produced by developing germ cells in testis and transits through luminal space to the IS of epididymis to trigger its differentiation. The differentiated IS then secretes many proteins including proteases (including OVCH2) that act on sperm ADAM3 to make spermatozoa fully functional. As various genes are down-regulated in *Nell2* KO caput epididymis (fig. S7), it is very likely that epididymal luminal factors other than proteases also regulate sperm maturation in a different way but downstream of NELL2-ROS1 lumicrine system. Likewise, because OVCH2 is required for male fertility, OVCH2 may process other sperm and/or epididymis proteins required for sperm maturation. The lumicrine concept that we have demonstrated here in the male reproductive tract may be applied more broadly to other organs in which luminal flow occurs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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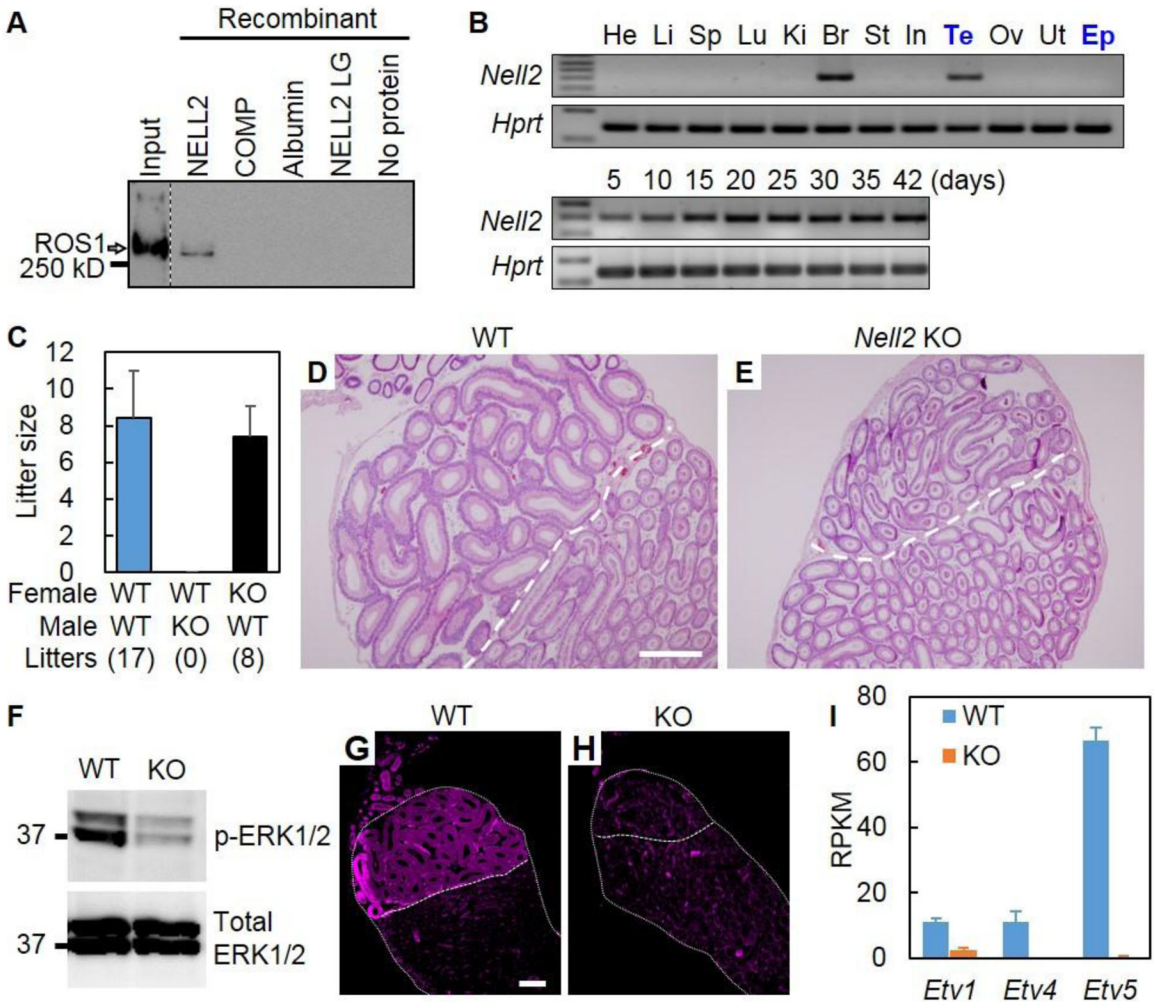

Fig. 1. Testicular NELL2 is indispensable for ROS1 mediated differentiation of epididymal initial segment.

(**A**) *In vitro* binding of ROS1 with NELL2 and several other proteins. (**B**) RT-PCR analyses of *Nell2* expression in adult organs and in postnatal testis. *Hprt* is also shown as internal controls. (**C**) Litter sizes of *Nell2* KO mice. Average and S.D. are shown. (**D**-**E**) HE staining of caput epididymis of 14w old WT (D) and *Nell2* KO (E) mice. Bars, 50 μm. (**F**-**H**) Phospho-ERK immunoblot (F) and immunofluorescence analyses of WT (G) and *Nell2* KO (H) 14w caput epididymis. Bars, 50 μm. (**I**) Expression of transcription factors downstream of ERK signaling in 14w WT and *Nell2* KO caput epididymis. RPKM, Reads Per Kilobase per Million.

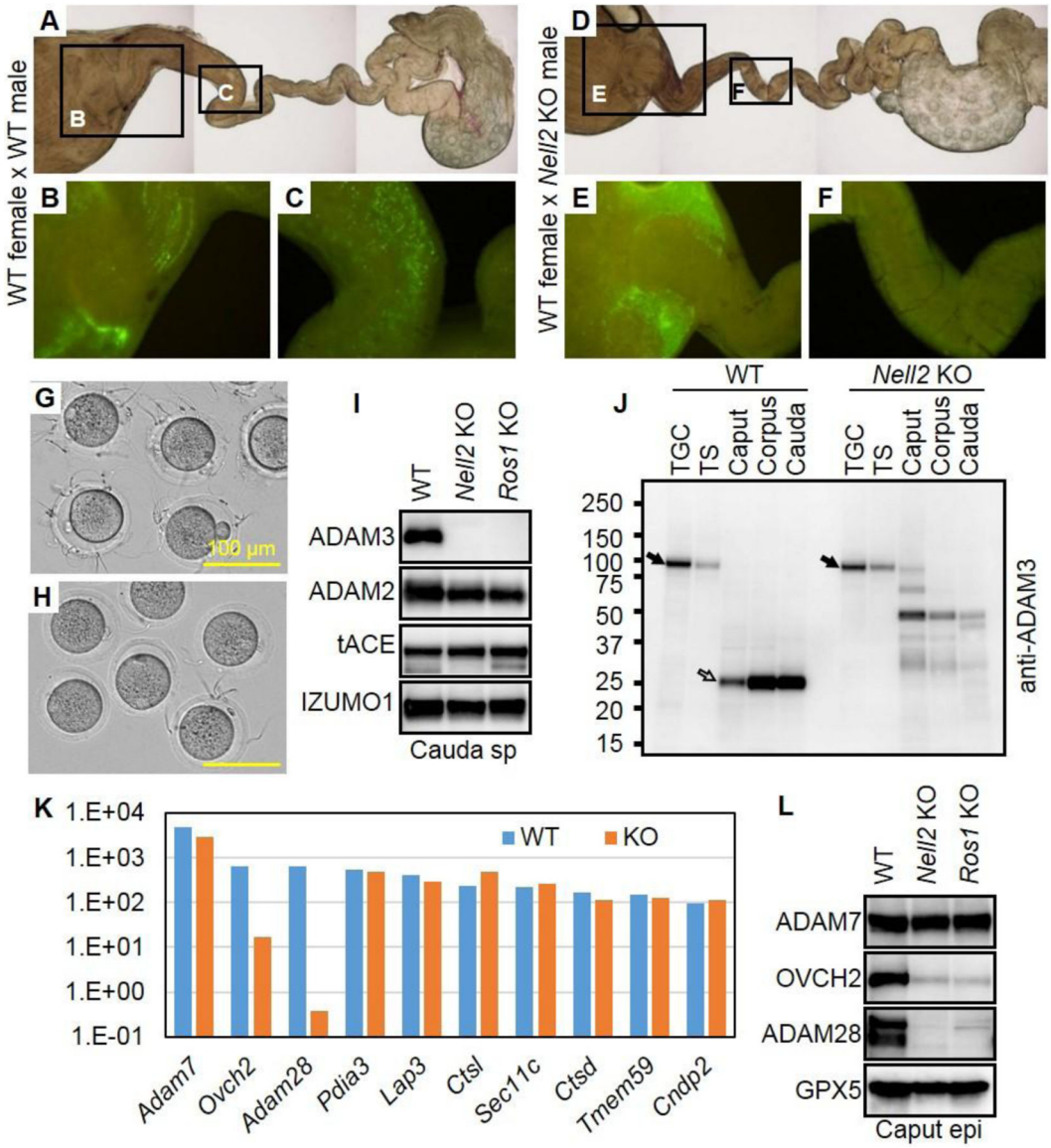

Fig. 2. NELL2 is required for ADAM3 maturation and subsequent sperm fertilizing ability.

(**A-F**) Migration of GFP-tagged WT (A-C) and *Nell2* KO (D-F) sperm ejaculated into WT female reproductive tract. (**G, H**) Sperm-ZP binding assay with WT (G) and *Nell2* KO (H) spermatozoa. Bars, 100 μm. (**I**) Immunoblot detection of cauda epididymal sperm lysates. (**J**) Immunoblot detection of ADAM3 in TGC, TS (testicular spermatozoa), caput, corpus, and cauda epididymal spermatozoa. (**K**) Expression of genes encoding proteases in WT and *Nell2* KO caput epididymis. Only genes whose average read counts in WT are >100 are shown. (**L**) Immunoblot analysis of caput epididymal protein lysates from WT, *Nell2* KO, and *Ros1* KO males.

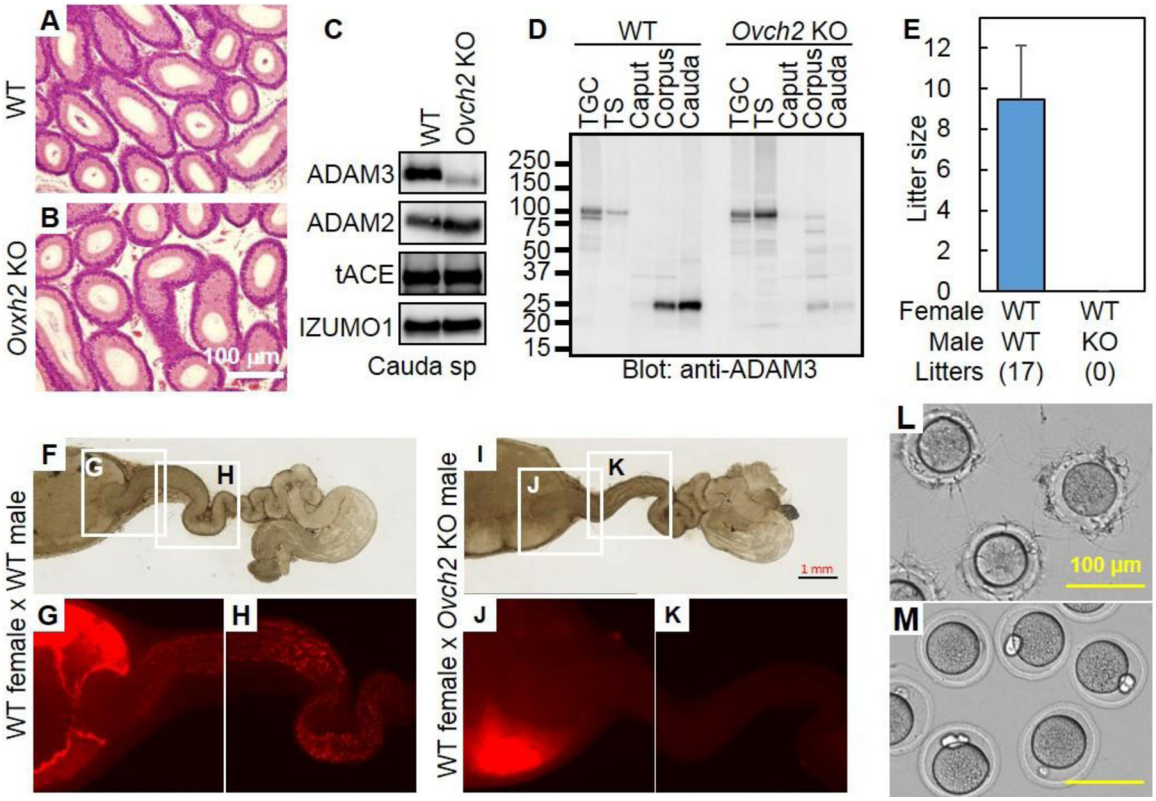

Fig. 3. Epididymal secreted protease, OVCH2, is required for ADAM3 processing and male fertility.

(**A, B**) HE staining of initial segment sections of epididymis from WT (A) and *Ovch2* KO (B) mice. Bar, 100 μm. (**C**) Immunoblot detection of cauda epididymal sperm lysates. (**D**) Immunoblot detection of ADAM3 in TGC, TS, caput, corpus, and cauda epididymal spermatozoa. (**E**) Litter size analysis of *Ovch2* KO mice. Average and S.D. are shown. (**F-K**) Migration of dsRed2-tagged WT (F-H) and *Ovch2* KO (I-K) spermatozoa ejaculated into WT female reproductive tract. (**L, M**) Sperm-ZP binding assay with WT (L) and *Nell2* (M) KO spermatozoa. Bars, 100 μm.

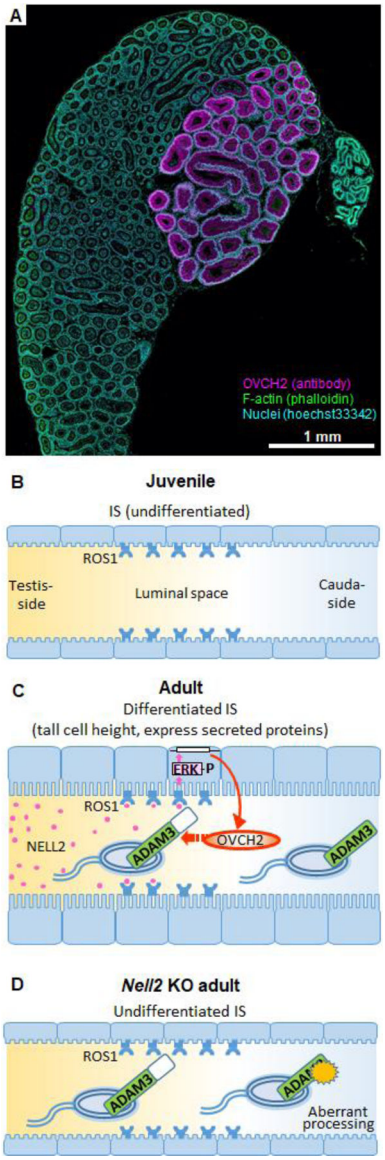

Fig. 4. Localization of OVCH2 in the caput epididymis and schematic lumicrine model representing testicular NELL2 regulation of epididymis-dependent sperm maturation.

(**A**) Precise localization of OVCH2 to the caput epididymis, the region most proximal to the testis. Staining of OVCH2 (magenta), F-actin (green), and nuclei (cyan) staining are shown. Bar, 1 mm. (**B**) In juvenile males, epididymal IS is undifferentiated. (**C**) During sexual maturation and in adult males, NELL2 is transported from testis to epididymis through the luminal space. IS epithelial cells are fully differentiated by the NELL2-ROS1-ERK signaling pathway and induces the secretion of proteases including OVCH2. OVCH2 subsequently promotes sperm surface ADAM3 processing, which is indispensable for sperm maturation. (**D**) In *Nell2* KO males, IS differentiation and OVCH2 protease secretion are impaired. As a consequence, ADAM3 processing is aberrant, and males are infertile.