A conditional Orco requirement in the somatic cyst cells for maintaining spermatids in a tight bundle in *Drosophila* testis

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Odorant receptors (OR) heterodimerizes with the OR co-receptor (Orco), forming specific odorant-gated cation channels, which are key to odor reception at the olfactory sensory neurons (OSN). Mammalian ORs are expressed in many other tissues, including testis. However, their biological implications are yet to be fully ascertained. In the mosquito, Orco is localized along the sperm tail and is indicated to maintain fidelity. Here, we show that orco expresses in *Drosophila* testis. The levels are higher in the somatic cyst cells. The *orco*-null mutants are perfectly fertile at 25°C. At 28°C, the coiled spermatid bundles are severely disrupted. The loss of Orco also disrupts the actin cap, which forms inside the head cyst cell at the rostral ends of the spermatid nuclei after coiling, and plays a key role in preventing the abnormal release of spermatids from the cyst enclosure. Both the defects are rescued by the somatic cyst cell-specific expression of the *UAS-orco* transgene. These results highlight a novel role of Orco in the somatic tissue during sperm release.

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1. Introduction

Chemo-sensation is essential for insect survival and propagation. It plays a key role in the finding of food, egg laying sites and mates, as well as in avoiding predators and potential dangers. The olfactory receptors and some of the gustatory receptors expressed in the olfactory sensory neurons (OSNs) are engaged in the reception of volatile components, called odorants. The olfactory receptors are also widely expressed in several other tissues (Kang and Koo 2012). In mammals, the receptor gene expressions are detected in testis, heart, brain, spleen, pancreas, tongue, lungs, kidneys and many more tissues (Kang and Koo 2012). In all these cases, expression of one or more receptors is documented. The exact function of many of these receptors outside the OSNs is still unclear. Ectopic expression and function of olfactory receptors such as MOR83, MOR8-1, MOR23, MOR127-5P and others, in the mammalian testis have been extensively studied (Vanderhaeghen *et al.* 1993; Walensky *et al.* 1995; Spehr *et al.* 2004; Neuhaus *et al.* 2006; Veitinger *et al.* 2011; Kang and Koo 2012). Mouse olfactory receptor, OR1D2 (MOR127-5P), localizes to the mid-piece of a sperm flagellum and helps in the chemotactic navigation of the sperm towards the egg (Vanderhaeghen *et al.* 1993; Spehr *et al.* 2004; Neuhaus *et al.* 2006; Veitinger *et al.* 2011).

Odorant receptors (ORs) are a unique family of seven-pass-transmembrane proteins found in insects (Clyne *et al.* 1999; Vosshall *et al.* 1999). They contribute to the reception of a major part of the odor repertoire (Malnic *et al.* 1999; Hallem *et al.* 2004), especially food and social odors. Each OSN expresses a single OR gene and responds to a particular set of odorants (Hallem and Carlson 2004; Hallem *et al.* 2004). The OR co-receptor (Orco), another unique seven-pass-transmembrane protein with a reverse topology to that of the GPCR-associated mammalian ORs, is expressed in all the OSNs (Larsson *et al.* 2004). Each OR heterodimerizes

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with the Orco, forming a ligand-gated cation channel (Larsson *et al.* 2004; Sato *et al.* 2008). Orco is also required for the OR localization to the olfactory cilia, an essential prerequisite for odorant reception by the OSNs (Benton *et al.* 2006). Upon an appropriate odorant binding to an OR/Orco complex, the cation channel opens up, depolarizing the OSN and generating an action potential (Neuhaus *et al.* 2005; Sato *et al.* 2008; Stensmyr *et al.* 2008).

The mosquitos *Anopheles gambea* and *Aedisaegypti* as well as the fruit fly *Drosophila melanogaster* belong to the order Diptera of the Culicidae insect family. Previous studies have indicated *orco* expression in the testes of these organisms (Pitts *et al.* 2014). AgOrco is localized along the spermatid tails, and treatment with the Orco agonist resulted in the activation and movement of the spermatozoa (Pitts *et al.* 2014). Interestingly, this report also mentioned Orco localization along the sperm tails in *Drosophila* testis, but its role in the sperm activation was not established.

In *Drosophila* testis, spermatogenesis takes place within a cellular enclosure formed by two somatic-origin cyst cells (CCs) (Fuller 1993; Fabian and Brill 2012). At the end of the process, it produces sixty-four, 1.8-mm-long mature spermatids that are tightly bundled within the CC enclosure. Subsequently, the spermatid bundle coils without disrupting the enclosure, and the entire cyst assembly moves into the terminal epithelium (TE) region at the testis base (figure 1A). The organization of the spermatid head bundle (also called the nuclei bundle and abbreviated as NB in this manuscript) undergoes a distinctive alteration in morphology during the coiling stages (figure 1A). In the end, sixty-four spermatid heads, arranged in the tight bullet-shaped bundle (NB), are encapsulated by the head cyst cell (HCC), and the tail bundle is wrapped around by a tail cyst cell (TCC). It was shown that the maintenance of the coiled spermatid bundle within the cyst enclosure is essential for a proper released from the somatic-cell enclosure (Desai *et al.* 2009). The tight organization of NB in the testis is maintained by F-actin-rich structure called actin cap. Actin caps are formed within the HCC, and they help in holding the spermatid heads together in a bundle resulting in the characteristic NB morphology. Disruption of the actin cap is associated with the NB disorganization (Desai *et al.* 2009).

Here, using a Gal4 expression screen and testis-specific RT-PCR analysis, we show that *orco* is expressed in the testis. Although the expression is quite prominent in the CCs at all stages, the *orco* knock-down in the CCs caused widespread disruption of only the coiled stage spermatid bundles. Furthermore, a similar phenotype, observed in the homozygous *orco* mutant testes at the elevated growth temperature, was rescued by the *orco-Gal4>UAS-orco* expression. Also, the actin cap morphology was disrupted in the homozygous *orco* mutant testes. Together, these results highlighted a novel role of Orco in the CCs during spermiation in *Drosophila*.

2. Materials and methods

2.1 *Drosophila stocks and culture condition*

All fly stocks were maintained on standard cornmeal-sucrose medium at 25°C. Canton-S was used as the wild-type control in the study. Freshly emerged males were allowed to attain maturity for 4 days in isolation from the females and transferred to vials containing fresh food every 12 h. To analyse the distribution of total intact NBs in a testis, batches of 10–20 freshly emerged males from the wild-type control, homozygous mutants and the genetically rescued stocks were shifted to 25°C and 28°C, and maintained for 4 days with a fresh change of food every day. Subsequently, the testes were dissected for staining. For the RNAi experiments, the crosses were set at 25°C and the freshly emerged males were shifted to 28°C for 4 days before staining. We noted that the NB organization were unstable, even in the wild-type control, when they were grown at higher temperature. After some permutations, we found that a regular change of the media minimized the defects. Therefore, all experiments were conducted by maintaining the flies under the same standard conditions. The sources of the fly stocks used in the study are mentioned in supplementary table 1. For fecundity experiments, *Canton-S* and $orco^{1}$ mutant males were grown at 25°C and aged for 4 days at 28°C in isolation from females. Subsequently, each male was mated with three Canton-S virgins for a day and the number of F1 progenies emerged from the cross was counted as a measure of fecundity.

2.2 *Whole mount immunostaining*

The testes from appropriately aged flies were dissected in Phosphate Buffer Saline (PBS) and then fixed for 1 h in 4% paraformaldehyde (PFA) made in PBS at room temperature. The specimen were then washed three times with PBT (0.3% Triton-X in PBS). This was followed by an overnight incubation with the primary antibody in PBT at appropriate dilutions. Subsequently, the samples were washed three times in PBT, incubated with appropriate secondary antisera conjugated with the Alexa-dyes for 2 h at room temperature, and mounted with a drop of anti-fade media after a brief wash in PBT. For the NB and actin cap staining, the testes preparations were briefly incubated for 30 min in 1μg/mL Hoechst dye in PBT and 10 μM $Phalloidin^{Atto\text{-}565}$. The specimen were then washed in PBT and mounted in Vectasheild™ mounting medium (Vector Labs, USA) on a slide for imaging.

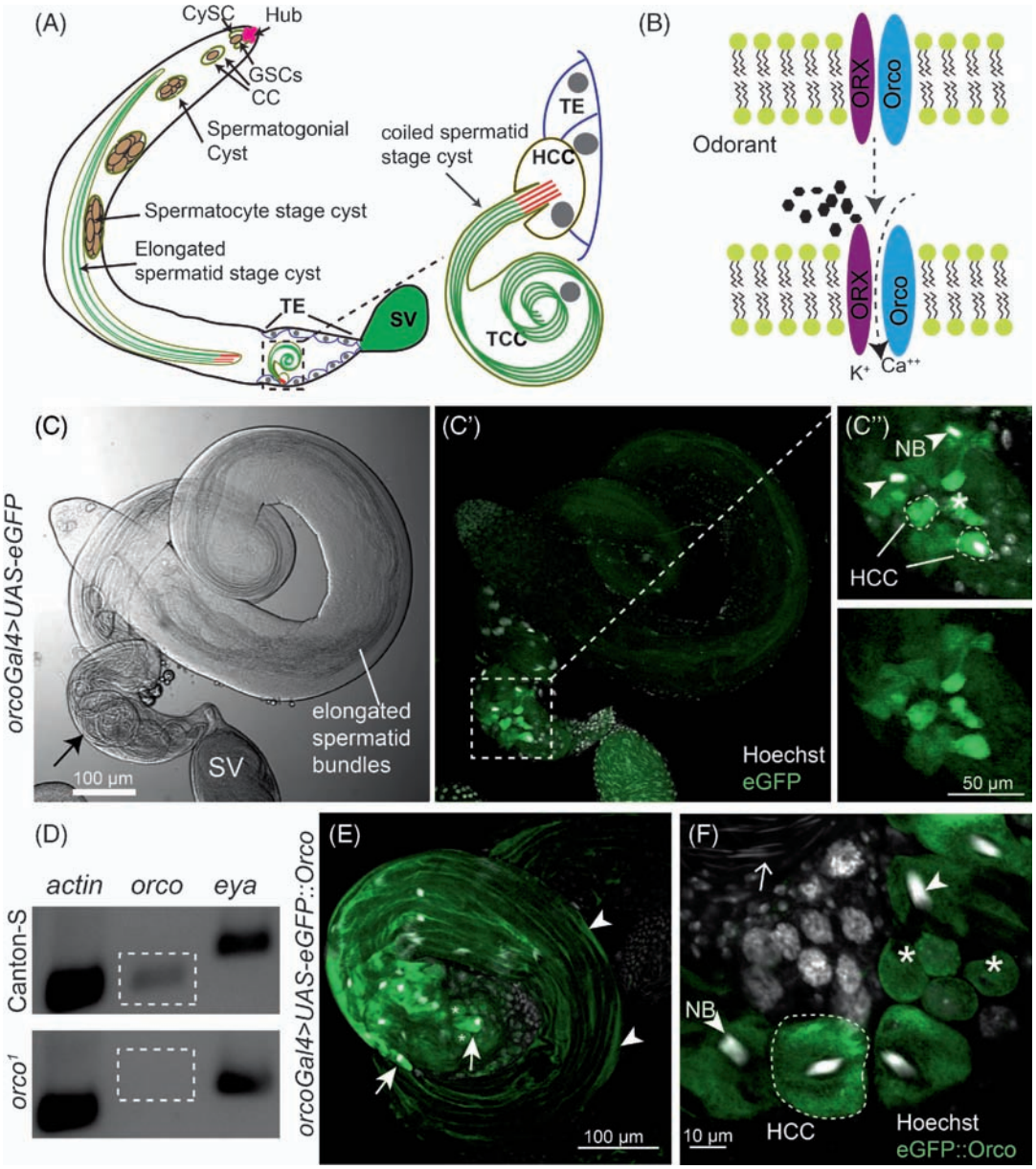


Figure 1. Expression of the olfactory receptors co-receptor (orco) in *Drosophila* testis. (**A**) Schematic depicts different stages of spermatogenesis in *Drosophila* testis. The heads (red) and tails (green) of elongated, mature spermatids are depicted in distinct colours. Mature spermatids coil up inside the somatic cyst cells (HCC, TCC) at the base of the testis and the entire assembly attaches to the terminal epithelium before release. (Hub – hub cells, GSC – germline stem cells, CySC – somatic cyst stem cells, CC – Somatic Cyst Cell, HCC – head cyst cell, TCC – tail cyst cell, TE – terminal epithelium, SV – seminal vesicle) (**B**) Schematic illustrates the mechanism of olfactory signal transduction by Odorant receptor (ORx) and OR co-receptor (Orco) complex. (**C**–**C′′**) The *orco-Gal4>UAS-eGFP* expression in the 2-day-old testis. (**C**) DIC image depicts the distribution of coiled stage spermatids at the base (arrow, **C**) and GFP (green) fluorescence represents the *orco-Gal4* expression pattern (**C′**). The enlarged views of the boxed region of the testis base shown in (**C′′**) illustrate the expressions in HCCs encapsulating the coiled-stage spermatid heads (NBs, arrowheads) and the adjacent cells (*). (**D**–**F**) Orco expression in the adult testis. (**D**) RT-PCR amplification of *actin*, *orco* and *eya* mRNAs from the extracts of 2-day-old Canton-S and homozygous *orco*^{1} mutant testes. The eyes-absent (*eya*)-specific primers were used as positive control. (**E**) Testes from 2-day-old *orco-Gal4>UAS-eGFP::orco* adults stained with Hoechst (white) highlight the expression and localization of eGFP::Orco (green) in the CCs of testis (arrows). (**F**) Magnified images of the basal region of the *orco-Gal4>UAS-eGFP::orco* testis show the recombinant Orco localization inside the late stage HCC and the adjacent cells (*).

2.3 *RT-PCR*

RNA from testes of 2-day-old Canton-S and homozygous *orco*[1] mutant adults were isolated with a Trizol reagent (ThermoFisher Scientific, USA). The cDNA was prepared from the isolated RNA samples using SuperScript® III RT (ThermoFisher Scientific, USA) and random hexamer primers. The PCR- amplification was performed with the set of primers as listed in supplementary table 2. The PCR reaction mixture was separated by electrophoresis in 1% low melting agarose gel and the bands were visualized using SYBR™ Safe (ThermoFisher Scientific, USA).

2.4 *Image acquisition and analysis*

Images were acquired using theUPLSAPO20X;NA-0.75 objective fitted on an Olympus FV1000SPD confocal, laser-scanning microscope. Post-acquisition images were analysed using the ImageJ. Statistical significance or the P-values were calculated using the Mann-Whitney U test.

3. Results

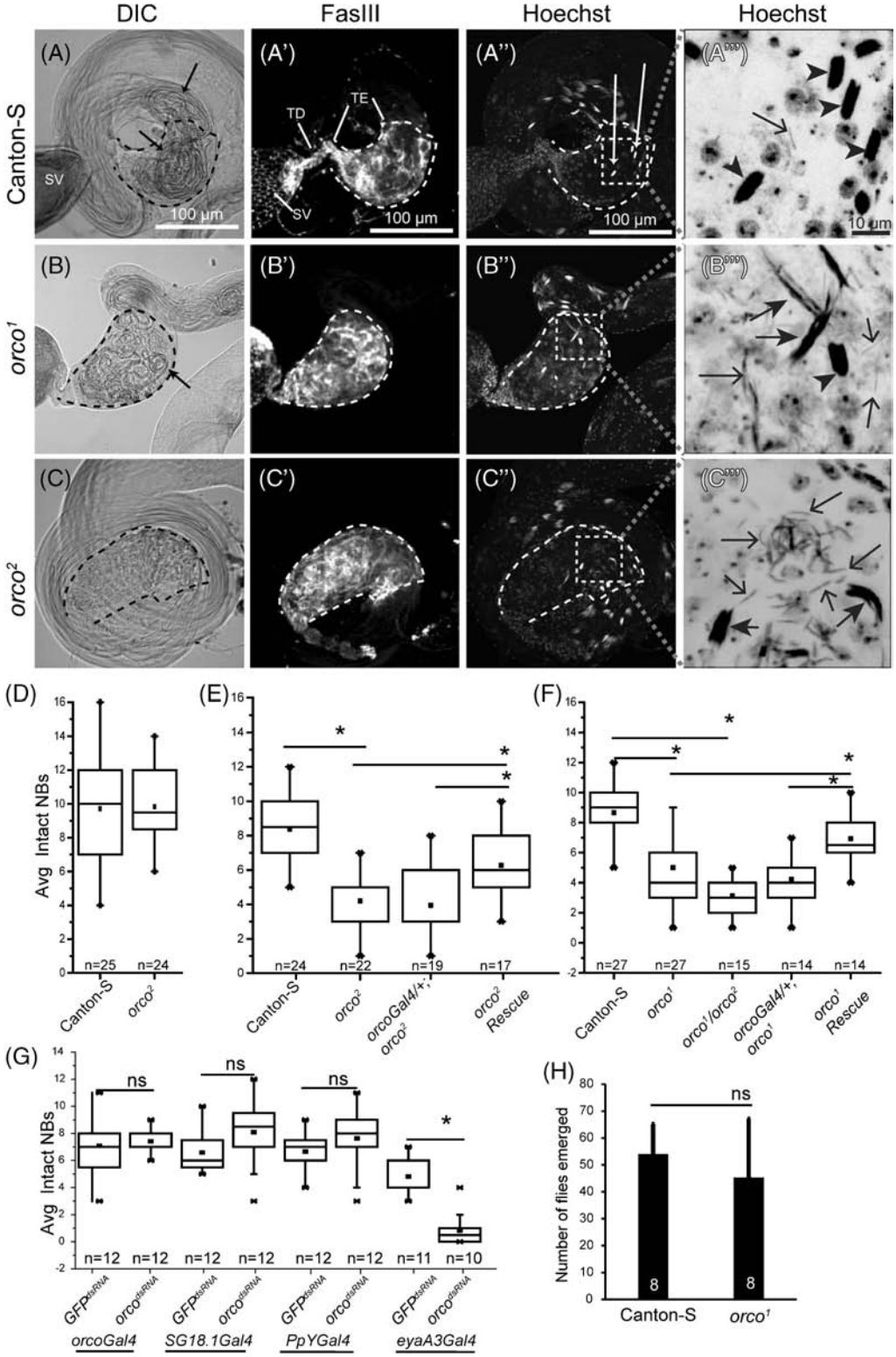
3.1 *orco expresses in the adult testis*

To identify the role of Orco during spermatogenesis, we first studied its expression in the testis. We crossed *UAS-eGFP* transgenic stock to *orco-Gal4* and noted the expression patterns in the testis of the resultant progeny. This indicated that *orco* mRNA is likely to express in the testis (figure 1C–C′′). The signal was particularly prominent in the HCC during the coiled stages of the spermatogenesis (figure1C′′). It also marked some somatic cells adjacent to each HCC (*, figure 1C′′) whose identity is unclear. To confirm whether the *orco-Gal4* expressions indeed represents that of the corresponding endogenous mRNA, we amplified the *orco* cDNA from the testis-specific total mRNA by RT-PCR using a limited set of *orco*-specific primers. It amplified an expected size band from the Canton-S testis (figure 1D), which was absent in the total mRNA extract of the homozygous *orco*[1] testes. Altogether these data confirmed that the *orco* is indeed expressed in adult testis.

3.2 *Orco expression is elevated in the head cyst cell during the late stages of spermatogenesis*

Orco forms homodimeric as well as the heteromeric channels with other ORs. Thus, it is essential for the functioning of all ORs. Also, Orco plays a critical role in sperm activation in *A. Gambea* (Pitts *et al.* 2014). The AgOrco is reported to localize along the sperm tail (Pitts *et al.* 2014). However, we observed an elevated expression of GFP from the *orco* promoter in the somatic-origin cyst cells (CCs) in *Drosophila* testis (supplementary figure 1). Therefore, to further analyse Orco expression and localization pattern, we expressed *UAS-eGFP::orco* using *orco-Gal4* (figure 1E–F). It highlighted a pattern similar to the one revealed by *orco-Gal4>UAS-eGFP*. The eGFP::Orco localization was restricted to the cytoplasm (figure 1F). Although the earliest expression was observed in the CCs surrounding the spermatocytes and the post-meiotic spermatids (supplementary figure 1), eGFP::Orco was most prominent in the CCs surrounding the elongated spermatids (arrowheads, figure 1E). The expression continued in the head cyst cells (HCC, arrows, figure 1E) surrounding the NBs of coiled spermatids. Also, it marked a few cells adjacent to the HCC in the TE region (*, figure 1F). The expression in CCs is consistent with the reported *orco* expression in insect testes (Pitts *et al.* 2014). Orco immunostaining was shown to mark the somatic encapsulation of the germline cysts at the

Figure 2. Orco loss causes conditional NB disruptions at the late stage before spermiation at non-permissive growth conditions. (**A–C**) Testes from 2-day-old the Canton-S (**A–A′′**), and homozygous *orco*[1] (**B–B′′**) and *orco*[2] (**C–C′′**) mutant flies were stained with the Anti-fasIII antibody (**A′–C′**) marking the terminal epithelium region (highlighted by broken lines), and the Hoechst dye (**A′′–C′′**) marking the nuclei. The enlarged images of the boxed regions in (**A′′–C′′**) are depicted using an inverted LUT in panels (**A′′′–C′′′**). The DIC images of the testes show the organization of coiled spermatids (fine arrows, **A** and **B**) at the base of the testes. The Hoechst staining highlight intact (long arrows and arrowheads) and disrupted (arrows) NBs, as well as dispersed sperm heads (open arrows) at the testis base. (**D–F**) Box plots show distribution of intact NBs at the TE region of the testes from Canton-S, homozygous *orco*[1] and *orco*[2], *orco*[2] rescue (*w; orco-Gal4/UAS-eGFP::orco; orco*[2]), *orco*[1] rescue (*w; orco-Gal4/UAS-eGFP::orco; orco*[1]), and *orco-Gal4* controls (*w; orco-Gal4/+; orco*[1] and *w; orco-Gal4/+; orco*[2]) used for the rescue experiments. (**G**) The box plots show the distribution of intact NBs at the TE region of the testis estimated in different genetic backgrounds as indicated at the lower margins. Freshly emerged males were isolated and kept at 28°C for four days before the fixation and staining. CC-specific expression of the *UAS-orco*[dsRNA] using the *orco-GAL4*, *SG18.1-Gal4*, *PpY-Gal4,* and *eyaA3-Gal4,* respectively, caused moderate to severe NB disruption. The *GFP*[dsRNA] was used as negative control in each case. All stocks were grown at 25°C until the adults emerge, and then, shifted to 28°C for four days before fixation. Only the plots in (**D**) were obtained from stocks maintained throughout at 25°C. (**H**) Histograms depict the average fecundity of Canton-S and homozygous *orco*[1]males after being grown for four days at 28°C. The P-values of the pair-wise significance of differences indicated on the paired bars (*, <0.01), were estimated using the Mann-Whitney U test, and samples sizes are depicted below each box. Each box indicates the distribution of the 25%–75% of data points with the median (dissecting bar) and the mean (central square) values marked on them.


DIC
FasIII
Hoechst
Hoechst
Canton-S
orco1
orco2
(A)
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(B')
(B'')
(B''')
(C)
(C')
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SV
TD
TE
100 µm
10 µm
(D)
(E)
(F)
(G)
(H)
Avg Intact NBs
Number of flies emerged
n=25
n=24
n=22
n=19
n=17
n=27
n=15
n=14
n=12
n=11
n=10
Canton-S
orco2
orcoGal4/+;orco2
orco2 Rescue
orco1
orco1/orco2
orcoGal4/+;orco1
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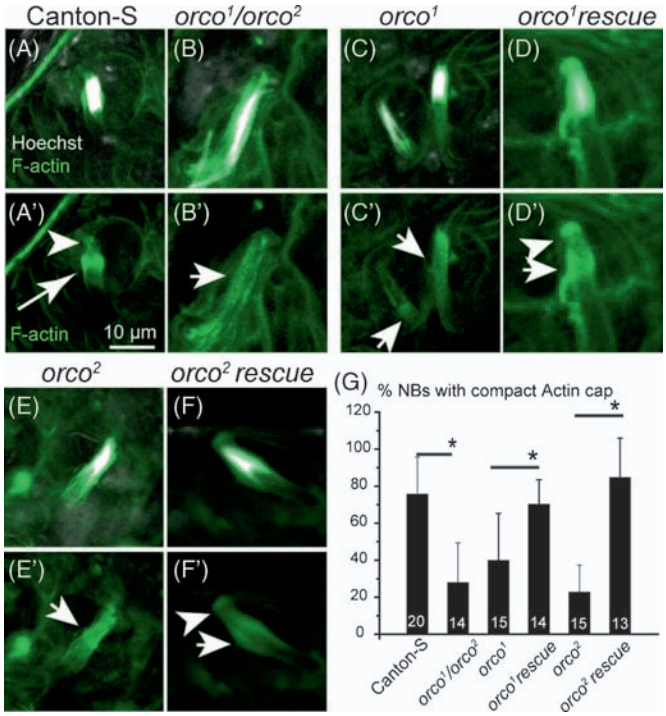

Figure 3. The actin cap organization is disrupted in the homozygous *orco* mutants. (**A–F**) The figure set show morphology of the NBs and associated actin caps in the testes obtained from adults of different genotypes grown at 28°C for 4 days after eclosion. The panels depict representative pictures obtained from the Canton-S (**A**), *orco*^{1}/*orco*^{2} (**B**), homozygous *orco*^{1} (**C**), *orco*^{1}*rescued* (**D**, *w; orco-Gal4/UAS-eGFP::orco; orco*^{1}), homozygous o*rco*^{2} (**E**) and *orco*^{2} rescued (**F**, *w; orco-Gal4>UAS-eGFP::orco; orco*^{2}), respectively.The arrows and arrowheads point towards the rostral and lateral parts of an actin cap recognized by the characteristic morphology. The morphology is disrupted in the homozygous *orco* mutants and restored in the corresponding transgenic rescue backgrounds. (**G**) Histograms depict % NBs (mean±S.D.) with compact actin cap morphology as depicted in the panel (**A–F**). The numbers on the bars indicate the number of testis examined. The pairwise significance of differences (P-values, *, <0.01) were calculated using the Mann-Whitney U test.

early stages in *A. Aegypti* testis (Pitts *et al.* 2014), although these authors did not comment on it. The staining pattern at the later stages was not discussed. The data presented here suggests that *orco* may express in the CCs throughout spermatogenesis.

3.3 *Orco function in the HCC is necessary for maintaining the spermatid bundle after coiling*

The *orco* expression is fully disrupted in the homozygous *orco*^{1}and *orco*^{2} mutants due to an insertion of the *mini-white* element in place of the first three exons (Larsson *et al.* 2004). The fecundity of homozygous *orco*^{1}adults was comparable to that of the Canton-S males, grown at 25°C. The morphology of homozygous *orco*^{1}and o*rco*^{2} testes also appeared normal. At 28°C, however, the organization of spermatid bundles at the testis base was visibly disrupted (figure 2B and C). The TE region of the testis, marked by FasIII immunostaining (figure 2B′ and C′), contained several disrupted NBs (arrows, figure 2B′′′ and C′′′) and dispersed sperm heads (open arrows, figure 2B′′′ and C′′′). Also, the number of intact NBs in the TE zone was significantly reduced in both the homozygous *orco*^{1} and *orco*^{2} mutants (figure 2E and F). Transgenic expression of *UAS-Orco* by *orco-Gal4* in the homozygous *orco*^{1} and *orco*^{2} backgrounds, respectively, rescued the defects (figure 2E and F). Thus, it is indicated that Orco function is required for maintaining spermatid bundles in the final stages of spermiation.

To test the somatic requirement of the Orco function in the maintenance of the spermatid organization, we expressed the *UAS-orco*^{dsRNA} transgene using four different Gal4 drivers (*SG18.1-Gal4*, *orco-Gal4*, *PpY-Gal4*, and *eyaA3-Gal4*). The expression due to the *SG18.1-Gal4* in the pre-meiotic germ cells and HCCs during the coiled stage did not cause a significant NB disruption (figure 2G). Also, no major NB disruptions were caused by the expressions due to *orco-Gal4* and *PpY-Gal4* as well (figure 2G), although the latter is exclusively expressed in the CCs from the post elongation stages (Jung *et al.* 2007). The *orco*^{dsRNA}

expression by the *eyaA3-Gal4*, which is strongly expressed in the CCs from the spermatocyte stage onwards, recapitulated the *orco* mutant phenotype (figure 2G). These results suggested a distinct role of Orco in the CCs in maintaining the NBs after coiling. We further investigated whether these defects can lead to a decrease in the fecundity of the homozygous mutant males. No significant difference in the fecundity between Canton-S and homozygous $orco^{1}$ mutant males were detected after 4 days at 28°C (figure 2H).

Together, the conditional loss of NB organization at a higher temperature in homozygous *orco* mutants, and the NB disruption due to the $orco^{dsRNA}$expression by a strong, CC-specific promoter element, suggested that Orco function is required in the somatic tissue for preventing premature spermiation at elevated growth temperature. Although the cellular defects induced by the loss did not appear to affect fecundity in the near term, a longer-term loss of fecundity with aging in the mutants is not ruled out.

3.4 *Loss of Orco also disrupts actin caps inside HCC*

The NB of mature spermatids was inserted into the HCC at the coiled stage (Tokuyasu *et al.* 1972). In our previous study, we have shown that F-actin-rich membrane extensions of HCC encapsulate the NB, and the ensemble is called actin cap (Desai *et al.* 2009). Depolymerisation of F-actin, using Latrunculin B, dissolved the actin cap, leads to disruption of the tightly packed NBs as well as the spermatids. Also, CC-specific disruption of *dynamin* (*shibire)* function disrupted the actin cap, and it was associated with NB disorganization (Desai *et al.* 2009). Together, these data established that the formation and maintenance of actin cap are essential for maintaining the spermatid bundle integrity. The NB disruption observed in the homozygous *orco* mutants resembled the phenotypes caused due to actin cap perturbation, described in the earlier report. Therefore, we investigated the actin cap morphology in the homozygous *orco* mutant testes.

Usually, the actin cap forms around an NB and extends along the entire length (arrow, figure 3A–A′). It also contains a distinctive rostral extension (arrowhead, figure 3A–A′). We defined this as the compact morphology of the actin cap. The actin caps in the homozygous $orco^{1}$ (figure 3C) and $orco^{2}$ (figure 3E), as well as in the $orco^{1/2}$ (figure 3B) transheterozygous, mutants were relatively less organized as compared to those in the wild-type controls (Canton-S, *orco* rescue, figure 3A and F). Often, the actin caps lacked the regular compact morphology and the distinctive rostral extension (figure 3B, C and E). Counts of the intact NBs associated with a compact actin cap suggested that the number is significantly lower in the homozygous *orco* mutant testes (figure 3G), and the defect was rescued by the ectopic expression of the *UAS-eGFP::orco* transgene in the somatic tissue (figure 3G). These results further highlighted a novel role of Orco in maintaining the actin caps inside the HCC.

4. Discussion

Our study reports expression and a role of the olfactory co-receptor (Orco) during *Drosophila* spermatogenesis. Using RT-PCR and *orco-Gal4* expression analysis, we showed that *orco* mRNA is expressed in the adult testis. Also, it is likely to be enriched in the HCC during the coiled-stage cyst. Further, using homozygous *orco*-null mutants and the transgenic rescue stocks, as well as the CC-specific *orco* knockdown, we demonstrated that Orco is required in the CCs in maintaining the integrity of the actin cap and coiled spermatid bundles before release. The defect only manifested at an elevated growth temperature, suggesting a conditional requirement of Orco during spermiogenesis. The phenotype is relatively benign and does not significantly affect the fecundity of young adults even at elevated temperature. All previous studies using the *orco* mutants were carried out at an ambient temperature, and the homozygous *orco* mutant adults are perfectly fertile at 25°C. In the mosquito, AgORs are localized along the sperm tails, and they are predicted to play an important role in sperm activation. Pitts *et al.* (2013) also observed the Orco localization along the *Drosophila* spermatid tails. However, the functional significance of this localization was not discussed. The above data suggests yet another novel role of Orco in the CCs, which possibly helps in maintaining proper spermiation under stressful growth conditions.

Unlike the previous reports, we failed to detect Orco expression in the spermatids using the promoter-Gal4 fusion elements. All the transcriptional activity and a majority of the translational activities in the spermatids are stopped after elongation when the nuclear DNA is super-compacted by replacing Histones with the Protamins (White-Cooper 2010). Therefore, *orco-Gal4* screen is unlikely to report expression in the spermatids in the later stages. A large number of promoter-Gal4 fusion elements were also found to express in the HCC during the coiled stage, indicating a general tendency of the Hsp70 TATA box to drive expressions in this tissue (Dubey and Ray, unpublished). Therefore, we explored the functional requirement of Orco by expressing $orco^{dsRNA}$ in the CCs, as well as by the genetic rescue of the mutant phenotypes due to the *UAS-eGFP::Orco* expression in the somatic tissue in the homozygous $orco^{1}$ and $orco^{2}$ backgrounds. The possibility of an unrelated mutation in the background causing the defects was ruled by the analysis in $orco^{1/2}$ trans-heterozygous background. Together, the results confirmed a requirement of Orco in the somatic tissue, particularly in the HCC at the late stages of spermiation.

In a recent study, we observed that the sperm release is a passive process, and the actin cap plays a significant role in

preventing abnormal penetration of the HCC (Dubey *et al.* unpublished). Here, we showed that the actin caps are somewhat deformed in the homozygous *orco* mutant testes. A similar morphology was observed when the Dynamin function was disrupted in the HCC (Desai *et al.* 2009). Thus, this indicates that the Orco function is needed in the HCC to prevent an abnormal penetration of the cell by the coiled-up spermatids. The defects were conditional and manifested at an elevated growth temperature. Therefore, we concluded that the Orco function in the HCC is possibly required to mitigate the abnormal release under stressful growth condition.

The OR/Orco complex forms a functional cation channel allowing both the potassium and calcium influx upon odorant binding (Neuhaus *et al.* 2005; Sato *et al.* 2008; Stensmyr *et al.* 2008). MOR23, the mammalian OR, localized to sperm tails, regulates the sperm flagellar movement by altering the calcium dynamics (Fukuda *et al.* 2004). In mosquitos, the *AgOr*-dependent sperm activation is proposed to be mediated by calcium signalling (Pitts *et al.* 2014). These studies highlight a role of the olfactory receptor-mediated calcium signalling as a key functional event guiding sperm motility.

Transient and localized change in the calcium ion (Ca^{++}) concentrations regulate many cellular processes (Clapham 2007), including the F-actin dynamics (Janmey 1994). The stimulation of the actomyosin contractility (Tan and Boss 1992), activation of the F-actin severing proteins (Yamamoto 1982), inhibition of the α-actinin (Fechheimer *et al.* 1982; Witke 1993), and stabilization of the F-actin filaments (Lin and Redmond 2008) are some of the key functions regulated by Ca^{++}. A majority of the actin caps in homozygous *orco* mutant testes appeared elongated even when the NBs were intact. This evidence suggested that the Orco function is needed for maintaining the F-actin organization in this region. According to our latest observations, the formation of actin cap and the peak *orco-Gal4* expression phase in the coiled stage HCC coincides with the period when the actin cap is most dynamic. Further experiments are needed to establish a functional correlation between the Orco and the F-actin dynamics at the actin cap, as well as to understand its actual function. An Or-Gal4 expression screen and a preliminary RT-PCR analysis further suggested that several other ORs are also expressed in the testis (Dubey and Ray, unpublished). The contemporary expressions of both the Orco and some ORs in the testes and the *orco* mutant phenotypes indicate that the food and social odors could directly regulate the sperm release. Functional analysis with more OR mutants and downstream signalling molecules would reveal how ORx-Orco complex influence the spermiation at a cellular level.

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