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Olig2 regulates terminal differentiation and maturation of peripheral olfactory sensory neurons

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Abstract

The bHLH transcription factor Olig2 is required for sequential cell fate determination of both motor neurons and oligodendrocytes and for progenitor proliferation in the central nervous system. However, the role of Olig2 in peripheral sensory neurogenesis remains unknown. We report that Olig2 is transiently expressed in the newly differentiated olfactory sensory neurons (OSNs) and is downregulated in the mature OSNs in mice from early gestation to adulthood. Genetic fate mapping demonstrates that Olig2-expressing cells solely give rise to OSNs in the peripheral olfactory system. *Olig2* depletion does not affect the proliferation of peripheral olfactory progenitors and the fate determination of OSNs, sustentacular cells, and the olfactory ensheathing cells (OECs). However, the terminal differentiation and maturation of OSNs are compromised in either *Olig2* single or *Olig1/Olig2* double knockout mice, associated with significantly diminished expression of multiple OSN maturation and odorant signaling genes, including *Omp, Gnal, Adcy3,* and *Olfr15.* We further demonstrate that Olig2 binds to the E-box in the *Omp* promoter region to regulate its expression. Taken together, our results reveal a distinctly novel function of Olig2 in the periphery nervous system to regulate the terminal differentiation and maturation of olfactory sensory neurons.

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Keywords

basic helix-loop-helix (bHLH) transcription factors; peripheral nervous system (PNS); Tuj1; Sox2; Fabp7 (Blbp); Dcx

Introduction

Neuronal differentiation is a multi-step process involving cell fate specification, progenitor expansion, and the terminal maturation. Transcription factors, particularly, the basic helix-loop-helix (bHLH) transcription factors, are essential for the cell fate selection at the early-phase of neuronal differentiation (1, 2). However, it remains poorly understood whether or how bHLH transcription factors play a role in terminal differentiation or neuronal maturation process after neuronal cell fate determination. Olfactory sensory neurogenesis is an excellent model system to address this question. Generated from the basal progenitors, immature olfactory sensory neurons (OSNs) migrate a short distance toward the apical part of the olfactory epithelium (OE) where they express the mature OSN marker proteins and receptors and respond to odorants (3). Given the continuous turnover of OSNs in adult vertebrates and the requirement of the olfaction for sucking milk by neonatal rodents, proper terminal differentiation and maturation of the newborn OSNs is an essential step for functional olfaction and neonatal survival. Like in the central nervous system (CNS), a variety of bHLH factors, such as Ascl1 (Mash1), Ngn1 and Hes1, are identified in the olfactory progenitors and demonstrated to be important in controlling the proliferation and/or the differentiation of olfactory progenitors as they do in the CNS (4-7). However, the role of bHLH transcription factors in the later phase of OSN differentiation is poorly understood.

Olig2 is a bHLH family member originally identified as a crucial transcription factor for the sequential development of motor neurons and oligodendrocytes in the embryonic spinal cord (8-10). Later, it has been proved to control the proliferation of neural progenitors, and the genesis of cortical GABAergic neurons and white matter astrocytes (11-13). All support a proliferation-stimulating or gliogenic function of Olig2 in CNS. In the present study, we explored the role of Olig2 in the differentiation of OSNs and found that Olig2 is dispensable for the proliferation and fate determination of the OE progenitors, but it is specifically involved in the final maturation of OSNs, partially by regulating the expression of multiple genes, including *Omp, Gnal, Adcy3,* and *Olfr15,* which encode critical proteins for OSN maturation or odorant transduction.

Materials and methods

Genetically modified mouse lines

The *Olig2*^{CreER} knock-in mice, *Olig1/Olig2* double knockout, and *Olig2-Cre* mice were previously described (9, 10, 14). *Rosa26-LacZ* mice (15) were acquired from the Jackson Laboratory. All animal experiments were carried out under protocols approved by UC Davis Animal Care and Use Committees and following NIH guidelines. Pregnant, timed mated

mice were euthanized prior to cesarean section. The noon of the conception day was designated as E0.5.

Immunohistochemistry and BrdU labeling

Immunohistochemistry was conducted as described (16). The embryos were immersion-fixed in 4% paraformaldehyde (PFA). The postnatal and adult mice were transcardially perfused and fixed by 4% PFA. 30% sucrose was used for cryoprotection, and 12~14 μm frozen sections were made by a cryostat. Air dried slides were permeabilized by 0.3% Tween-20 in PBS, blocked by 10% lamb serum, incubated with primary antibodies at 4°C overnight. After washing in PBS, the corresponding secondary antibodies (Alexa Fluor 488 or 594 conjugated anti-rabbit, anti-mouse, anti-guinea pig, or anti-goat, 1:500, Invitrogen) were incubated for 2 hours at room temperature. Cell nuclei were stained by DAPI. The primary antibodies and dilutions are as following: rabbit anti-Olig2 (1:200, Millipore), mouse anti-Sox2 (1:50, Cell Signaling Technology), rabbit anti-Fabp7 (BLBP) (1:1000, Millipore), mouse anti-BrdU (1:50, Developmental Studies Hybridoma Bank), guinea pig anti-Dcx (1:1000, Millipore), and goat anti-OMP (1:1000, Wako). For acute BrdU labeling, pregnant mice were intraperitoneally injected with 10 mg/kg BrdU. Embryos were sampled after 1-hour BrdU incorporation. Frozen sections were treated by 2N HCl to denature DNA at 37°C for 20 min, and by 0.1M borate sodium buffer (pH 8.5) to neutralize the sections for subsequent immunohistochemistry with BrdU antibodies.

RNA *in situ* hybridization—Wholemount and section *in situ* hybridization were conducted as described (16). RNA probes for *Omp, Gnal (Golf), Adcy3 (AC3), Lhx2,* and *Ebf1 (O/E-1)* were made by EST clones. Other probes were made by PCR-based *in vitro* transcription using primers listed on Allen Mouse Brain Atlas website, and also shown below. *Olig2*: forward 5'-*AATATGGGAACCGAAGCAATG*-3', reverse 5'-*GCTCCTGTGCTCTGAAAAGG*-3'; *Ncam1*: *CAGGTAGATATTGTTCCCAG*, *GTCCTTGAAGTTGATTTCCC*; *Gap43*: *GGCTCTGCTACTACCGATGC*, *GCAGGAGAGACAGGGTTCAG*; *Olfr15*: *GGCCTCTTACTTGTTGACGC*, *ATGACGCTTACTGGGACCAC*; *Mecp2*: *AGACAAGCCACTGAAGTTTAAGAAG*, *TTGACAACAAGTTTCCCAGG*; *Arfgef2*: *CGAGCAAGGAACACTCAACA*, *TGTTTGGACCATGCAGACAT*; *Kirrel2*: *GCTTGGTTTCCACTCAGCTC*, *CAGCAAAGGAAAACGAGGAG*. Sections were counterstained by nuclear fast red.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted according to the manual of the ChIP assay kit (Upstate) and a slightly modified protocol (17, 18). Briefly, OE of E18.5 *wild-type* mice were dissected and fixed by 4% PFA for 15min on ice. Sonication was performed to break chromatin. Rabbit anti-Olig2 (1:100, Millipore) was incubated with DNA-protein complex overnight at 4°C. Rabbit IgG at the same concentration was used as the negative control. The primer pairs targeting the E-boxes in the *Omp* promoter were: (1) *GTGGTTCAGTTACAGAGCCC, AGAAACCCTCCTGCTTGAGC;* (2) *TATGTGGTTGGATCGATCAAAC, TTATCACCATCAGGACCCAG;* (3) *AACAAACAAATAGAACAGAGCAGGC, ATTGCCAGATGGAGGTCAAC;* (4) *TGTGTGTGTGTGTGATGTTC,*

TGTATGTGGACAGATGGCAG; (5) *CCGTCTGTCTGGCAGATGATTTG, TCATAGCCCCTGTCAGGTCC.*

Luciferase reporter assay

The promoter region of *Omp* was PCR amplified from the mouse genomic DNA. Based on the ChIP data, three fragments of *Omp* promoter were cloned into *pGL2-basic* vector (Promega, Madison, WI) to construct *Luci-Omp1* (containing E-box 1, or E1), *Luci-Omp1-3* (containing E1-3 boxes), *Luci-Omp1-6* (containing E1-6 boxes), *Luci-Omp4* (containing E4 box), and *Luci-Omp6* (containing E6 box) (Fig. 8A). Luciferase reporter assay was conducted as described (19, 20). Briefly, human embryonic kidney HEK293 cells were cultured to around 80% confluence. *Luci-Omp1, Luci-Omp1-3, Luci-Omp1-6, Luci-Omp4,* and *Luci-Omp6* were co-transfected with *pcDNA3.1-Olig2* using lipofectamine 2000 for 24 hours. PCR based mutation was performed to construct *Luci-Omp1-mutant.* Luciferase activity of total cell lysates was measured using Dual-luciferase reporter assay system (Promega). The *Renilla*-luciferase reporter *pRL* vectors (5 ng/well) was used as an internal control.

Real-time PCR

OEs of E18.5 embryos were dissected under microscope. RNA was extracted using Trizol reagent. cDNA was made by iScript™ cDNA Synthesis Kit (BIO-RAD). PCR was performed using SYBR GREEN PCR master mix (Thermofisher). The mRNA levels of *Fabp7* and *Omp* were normalized to the mRNA level of the house-keeping gene *Gapdh* to allow comparisons among different experimental groups using the Δ Ct method. The primer sequences are as following: *Omp, TCCGTCTACCGCCTCGATTT, CGTCTGCCTCATTCCAATCCA;* and *Fabp7 (Blbp), TTGATGAGTACATGAAAGCTCTGG, CTTGAATGTGCATTGTGTCC.*

Cell counting and statistical analyses

Cells positive for immunolabeling of Olig2, Sox2, Dcx, Omp, BrdU, and Fabp7; and *in situ* hybridization signals of *Omp, Gnal, Olfr15, Arfgef2, Kirrel2, Adcy3, Ncam1,* and *Gap43* were counted under microscope along OE lining the nasal septum from dorsal to ventral in three sections for each animal. The *in situ* hybridization "positive" cells were identified if the positive signal is localized in cytoplasm surrounded a nucleus which was counterstained by fast red. The littermate control and mutant OE sections were mounted on the same slide for subsequent *in situ* hybridization and immunohistochemistry under the same condition. OE length was determined by tracing the outline of the epithelium basal lamina using Slidebook software. For OE thickness, the vertical distance from the basal cell layer to apical surface were measured in 6 points with 100-μm interval along the nasal septum on each of three sections per embryo. At least three mutants and three littermate control embryos were included for each quantitative analysis. The morphological analysis was performed by a researcher blind to genotypes. The quantitative data was represented as mean ± SE (standard error). Two-tailed Student's *t* test was used for comparisons of the *wild-type* control vs. *knockout* samples. One-way ANOVA was used for three-group comparisons. $P \leq 0.05$ is considered significant. Related statistical details are included in the figure legends or results.

Results

Olig2 is expressed in newly differentiated Sox2(−);Tuj1(+) OSNs of the peripheral olfactory system

As soon as the nasal pit forms, Olig2 is expressed in the olfactory epithelium of E10.5 and E11.5 mouse embryos (Fig. 1). Considering the function of Olig2 in the CNS neural progenitors (21) and the basal localization of olfactory neural progenitors (22), we asked whether Olig2 is expressed by olfactory progenitors. Double fluorescent immunohistochemistry demonstrates that Olig2 positive cells are scattered in the early developing OE at E11.5, but they do not express Sox2, a marker for neural stem cells and progenitors (23), which are widely and uniformly expressed throughout the E11.5 OE on the same tissue section (Fig. 1D-F). By contrast, all Olig2 positive OE cells express the neuron-specific class III ß-tubulin Tuj1 (Fig. 1G-I). These unexpected results suggest that Olig2 is not expressed in the OE progenitors but in newly differentiated OSNs during early gestation.

From mid-late gestation, Olig2 positive cells distribute mainly in the basal part of OE, through postnatal ages to adult (Fig. 1B,C). The seemly localization changes of the Olig2 positive cells at different ages may reflect the cellular reorganization following OE development and maturation. In the late embryonic stage, the cellular organization of OE becomes similar to the adult OE. Therefore, we further examined the details of Olig2 expression in E18.5 OE. Double-immunolabeling of Olig2 with Sox2, which is expressed in olfactory stem cells of the basal OE and in sustentacular glial cells of the apical OE during late gestation and adulthood (16, 24), shows that the basal Olig2 positive cells are localized immediately atop the basal Sox2-positive cells and away from the Sox2 positive sustentacular apical OE (Fig. 2A). Same as in E11.5, no Olig2-positive cells express Sox2 in E18.5 OE (Fig. 2A). Double-immunolabeling of Olig2 with the immature and migrating OSN marker Dcx shows that approximately 60.91 ± 0.81% of Olig2 positive cells (counted 489 from total 3 embryos) are Dcx positive (Fig. 2B). Double immunolabeling with the mature OSN marker Omp shows that approximately 11.17 ± 1.39% of Olig2 positive cells (counted 403 from total 3 embryos) are Omp positive (Fig. 2C). These results suggest that Olig2 is transiently expressed in the newly differentiated OSNs and it is down-regulated following the progress of OSN maturation.

Dispensable role of Olig2 in the proliferation of olfactory progenitor and gliogenesis in OE

Because a key role of Olig2 in CNS is to instruct the motor neuron and oligodendrocyte lineage cell fate, and approximately 30% of the Olig2 positive cells in the basal part of OE are neither Dcx nor OMP positive (Fig. 2), we then asked whether Olig2 plays a role in gliogenesis or the proliferation of peripheral olfactory progenitors, and whether Olig1 plays a synergetic role with Olig2 in olfactory sensory neurogenesis. *In situ* hybridization demonstrates that *Olig1* is also expressed in the embryonic OE, with a similar expression pattern with *Olig2* (Fig. 3A,D). We further addressed aforementioned questions on *Olig2* single- and *Olig1/Olig2* double-deficient OE at E18.5 due to the neonatal lethality of these mutant mice. *Olig1* expression is not affected in the *Olig2*-deficient OE (Fig. 3B). Morphologically in general, the mutant OE looks normal in either single $Olig2^{-/-}$ or double $Olig1^{-/-}$;$Olig2^{-/-}$ mutants at E18.5 (Fig. 3B,C,E,F). There is no significant difference of

OE thickness between *Olig2*$^{-/-}$ (159.07 ± 11.35 μm) and *wild-type* (171.16±5.72 μm) embryos at E18.5 (n = 3 embryos for each genotype, student's t test, T value −0.046, P = 0.966). Acute BrdU incorporation and TUNEL staining are adopted to evaluate proliferation and apoptosis, respectively (Fig. 4A,B). Apical Sox2 and Fabp7 (Blbp) expression are used as glial-like cell markers for sustentacular and olfactory ensheathing cells (OECs), respectively (16, 24) (Fig. 4C,D). Unexpectedly, the results show no differences in all of these parameters between the *wild-type* and *Olig2*$^{-/-}$ OE at E18.5 (Fig. 4), indicating that Olig2 is neither involved in progenitor proliferation nor gliogenesis in the OE, at least at the embryonic stage.

OSN-restricted cell lineage of Olig2-expressing OE cells

In CNS, Olig2-expressing cells generate not only neurons and oligodendrocytes, but also white matter astrocytes and even reactive astrocytes after injury (25). We next examined whether Olig2 expressing cells in OE generate olfactory glial cells by genetic fate mapping. *Olig2-Cre* mice (14) were crossed with the reporter *Rosa26-LacZ* mice (15). X-gal and Fabp7 (Blbp) staining were performed at E14.5 and P7 (Fig. 5). The results show that, at both time points, only neuronal progeny is detected. X-gal staining is neither seen in the sustentacular cell layer nor the Fabp7 positive OECs (Fig. 5A-D). These results are in agreement with the restricted expression of Olig2 in the newly differentiated and Tuj1 positive OSNs (Figs. 1,2).

Requirement of Olig2 for the terminal differentiation and maturation of OSNs

To address the role of Olig2 in peripheral sensory neurogenesis, we analyzed OSN differentiation in *Olig2*$^{-/-}$ OE by examining the expression of several critical marker genes for OSN maturation and function. *In situ* hybridization shows that *Olig2* depletion does not affect the expression of a pan OSN marker *Ncam1* and an immature OSN marker *Gap43* (Fig. 6A,B). However, expression of the mature OSN marker *Omp* is significantly decreased in the *Olig2*$^{-/-}$ OE (Fig. 6C). Immunohistochemistry shows that regardless Olig2 depletion, Tuj1 positive OSNs are conserved in the E18.5 *Olig2*$^{-/-}$ OE (Fig. 6D). Consistent with the *in situ* hybridization result, Omp immunolabelling shows a notable reduction at the protein level (Fig. 6E).

We then examined critical genes associated with OSN odorant signaling functions. *Adcy3* (*AC3*) encoding the type III adenylyl cyclase is required for olfaction (26). *Gnal* (*Golf*) encoding a stimulatory G protein alpha subunit is required for odorant signaling and postnatal survival (27). Both *Adcy3* and *Gnal* mRNAs are dramatically diminished in the *Olig2*$^{-/-}$ OE (Fig. 7A,B). Moreover, the number of olfactory sensory neurons which express olfactory receptor *Olfr15* is significantly decreased in *Olig2*$^{-/-}$ OE (Fig. 7C). These data indicate that the maturation and function of OSNs are impeded by Olig2 deficiency.

We next asked whether Olig2 selectively affects the maturation of certain subgroup of OSNs. According to the expression of axonal guidance factors, OSNs can be roughly grouped as Arfgef2 (Big2)-positive and Kirrel2-positive (28). *In situ* hybridization shows a similar reduction of both *Arfgef2* and *Kirrel2* positive cells, indicating a general role of Olig2 in the terminal differentiation of OSNs (Fig. 7D,E).

We further examined the expression of *Zfp423, Lhx2, Mecp2,* and *Ebf1* (*OE-1*), which encode transcription factors involved in the terminal differentiation of OSNs (29-32). No difference of the expression of all these factors are found between the *wild-type* and *Olig2*$^{-/-}$ OE by *in situ* hybridization (Fig. 8).

Considering that *Olig1* is expressed in *Olig2*$^{-/-}$ OE (Fig. 3), we asked whether functional redundancy between Olig1 and Olig2 may occur in the OE as in the spinal cord (9, 33). The OSN differentiation was then investigated in the *Olig1/Olig2* double knockout embryos (9) (Fig. 5). *In situ* hybridization of all aforementioned OSN marker genes in *Olig1/Olig2* double knockout OE demonstrates almost identical phenotypes as seen in the *Olig2* single knockout (Figs. 6-8), suggesting the requirement of Olig2, not Olig1 in terminal differentiation and functional maturation of OSNs.

Direct regulation of *Omp* expression by Olig2

To address the mechanism of Olig2 in regulation of critical OSN maturation genes, we conducted *in vitro* molecular biological analyses. As a bHLH transcription factor, Olig2 regulates its downstream factors by binding to the E-box sequences on the promoter of the target genes (34). Among the significantly down-regulated OE genes in the *Olig2*$^{-/-}$ mutants, Omp plays a crucial role in the final maturation of OSNs and also is a widely used early marker for mature OSNs (35, 36). Therefore, we choose *Omp* as an exemplary candidate of downstream target genes to address if it is directly regulated by Olig2. We searched the promoter region of *Omp* and found six E-boxes (E1, *CACCTG,* −534bp; E2, E3, *CAGATG,* −592bp, −623bp; E4, E5, *CATCTG,* −845bp, −1043bp; E6, *CACGTG,* −1247bp from *ATG* respectively) in the *Omp* promoter region (Fig. 9A). Chromatin immunoprecipitation assay shows that Olig2 bound to E1, E4 and E6 among six E-boxes, indicating that Olig2 may directly regulate *Omp* expression (Fig. 9B). Real time RT-PCR confirms that the *Omp* mRNAs are decreased significantly in the *Olig2*$^{-/-}$ OE (Fig. 9C). To identify which E-box may contribute to the Olig2-regulated *Omp* expression, we co-transfected *Olig2* full-length cDNAs with three *Omp* luciferase reporter constructs *(Luci-Ompl* containing E1, *Luci-Omp1-3* containing E1-3, and *Luci-Omp1-6* containing E1-6) (Fig. 9A). The results demonstrate that Olig2 significantly activates the transcriptional activity of all of these luciferase reporter constructs at the similar level (Fig. 9D), suggesting that E4 and E6 might be dispensable for Olig2-regulated *Omp* expression. Indeed, co-transfection of *Olig2* with constructs containing individual E4 (*Luci-Omp4*) or E6 (*Luci-Omp6*) shows no significant effects of both constructs on *Omp* expression as compared to *pGL2* control (Fig. 9E). On the other hand, the construct with mutated E1 box dramatically abolishes the Olig2-induced *Omp* transcription (Fig. 9F). Collectively, these results suggest that Olig2 directly regulates the *Omp* expression by binding to the E1 box, which might partially account for the compromised OSN maturation in the Olig2-deficient OE.

Discussion

The bHLH transcription factors have been well documented as cell fate instructors, but they are poorly studied in the late stages of neurogenesis. In the present study, we demonstrate a novel role of Olig2 in the terminal differentiation of sensory neurons by analyzing the cell

identity of Olig2 positive cells in the OE and the phenotypes of *Olig2* single and *Olig1/Olig2* double knockout mutants during peripheral sensory neurogenesis.

In CNS, Olig2 is expressed by neural progenitors from early embryonic stage. Our data shows that, Olig2 is expressed in the nascent olfactory placode as early as E10.5 in mice. Although distributed sparsely, Olig2 expression is restricted to the newly differentiated Tuj1 positive OSNs, without expression in the Sox2 positive OE progenitors at E11.5. The co-immunolabeling of Olig2 with Tuj1 has also been shown in rat E14.5 OE (37). In late gestation of mouse embryos, the Olig2 positive cells become layered and locate atop Sox2 positive progenitors in the basal cell layer. Our double immunolabeling of Olig2 with Dcx and Omp further reveals that it is expressed by the newly differentiated OSNs and down regulated with the maturation of OSNs. The neuronal lineage-restricted expression of Olig2 in newly differentiated OSNs and its absence in OE progenitors/glial lineage cells suggest a distinctly novel role of Olig2 in PNS development. Given that Olig2 positive cells give birth to both neurons and glia in CNS, the solely neuronal progeny of Olig2 fate mapping in OE is in line with its expression pattern, and further suggests a potential role of Olig2 in late stages of peripheral sensory neurogenesis.

To determine the new role of Olig2 in peripheral neurogenesis, we examined alterations of the proliferation, apoptosis, and differentiation of the OE in *Olig2*-deficient mutants. Because *Olig2-KO* mice die shortly after birth, we conducted analysis at E18.5. In line with the expression pattern and fate-tracing results, *Olig2* deficiency does not affect the proliferation of progenitors and the generation of glial cells in the OE. Interestingly, the expression of the pan OSN markers (Tuj1 and *Ncam1*) and immature marker (*Gap43*) remains unchanged, but the expression of multiple critical genes (*Omp, Gnal, Adcy3,* and *Olfr15*) required for OSN maturation and odorant signaling transduction is significantly reduced in *Olig2*-deficient OE.

Considering that Olig2 expression in the olfactory placode starts as early as E10.5, and the requirement of olfaction for the suckling behavior of neonatal pups, it is necessary that OSN maturation must occur before birth. Our data, therefore, suggest a key role of Olig2 in the maturation process of OSNs during embryonic development, in preparation of functional readiness of olfaction for neonatal survival in rodents. The neuronal maturation role of Olig2 is indirectly supported by a previous study reporting an essential role of another bHLH transcription factor NeuroD1 in terminal differentiation of subventricular derived neurons in the olfactory bulb, which act through different molecular mechanisms (38). Among the significantly down-regulated factors in *Olig2*-deficient OE as demonstrated in the current study, the GTP-binding G protein Golf (encoded by *Gnal*) and the type III adenylyl cyclase AC3 (encoded by *Adcy3*) are located in the olfactory cilia of the mature OSNs to transduce odorant signaling through the Golf-AC3-cAMP cascade (26, 39, 40). Together, these factors strongly support a novel role of Olig2 in functional maturation of OSNs during embryonic development.

The half reduction of *Omp* in *Olig2*$^{-/-}$ OE promoted us to assess if there is a redundant function of Olig1 with Olig2 in peripheral sensory neurogenesis. Indeed, *Olig1* is also expressed in embryonic OE in a similar pattern with *Olig2* expression. However, the similar

defects of OSN maturation in *Olig1/Olig2*-double knockout OE suggest a dispensable or minimal role of Olig1 in embryonic OE development, but its function remains to be determined in the future. Olig1 and Olig2 have been shown to play distinct roles in postnatal CNS development and disease (41). Therefore, it is highly possible that Olig1 and Olig2 also play different roles in postnatal and adult olfactory sensory neurogenesis in normal and pathological conditions and during regeneration processes.

The incomplete decline of OSN maturation in *Olig2*-deficient OE is quite intriguing, as both *Arfgef2* and *Kirrel2* positive subgroups of OSNs are diminished about 50%. It remains to be explored whether Olig2 is responsible for maturation of all or certain subgroups of OSNs, or other bHLH transcription factors may play a synergetic role with Olig2 in OSN maturation. Previous studies have reported that transcription factors Zfp423, Lhx2, Mecp2, and Ebf1 are involved in the terminal differentiation of OSNs (6, 29, 30, 32). However, the expression of these transcription factors remains unchanged in the *Olig2*-deficient OE, indicating that these transcription factors are not regulated by Olig2, but they may work with Olig2 together or in parallel to regulate OSN terminal differentiation and maturation. For instance, co-transfection of Olig2 and Nkx2.2 can induce oligodendrocytes, and co-transfection of Olig2 with HB9 but not Ngn2 can increase the motor neuron marker Isl1/2 from adult human olfactory epithelial-derived progenitors (42, 43). Olig2 and Sox10 induce oligodendrocyte differentiation through reciprocal interactions and dosage-dependent mechanisms in embryonic chicken spinal cord (44). The interactive and regulatory mechanisms of Olig2 with other transcription factors during sensory neurogenesis remain to be investigated.

During peripheral sensory neurogenesis, OSNs express several critical genes in the following sequence: from *Gap43* to *Adcy3* then *Omp* within 6 to 8 days after cell mitosis in postnatal and young adult mice (36, 45). Adcy3 acts downstream of Gnal for odorant signaling transduction (40). Omp plays a crucial role in the final maturation of OSNs and also is an early marker for mature OSNs (35, 36). Based on the results of unaltered *Gap43* and significantly diminished *Gnal, Adcy3,* and *Omp* expression in the *Olig2*-deficient OE, it is logical to propose that Olig2 directly or sequentially regulates *Gnal, Adcy3,* and *Omp* to promote OSN maturation. In the current study, chromatin immunoprecipitation and luciferase reporter assays demonstrate the direct regulation of Olig2 for one of these genes, *Omp.* It is highly possible that Olig2 also directly regulates additional downstream target genes, at least *Gnal* and *Adcy3* during OSN maturation. Therefore, how the expression of whole profile of the mature OSN-associated genes is regulated by Olig2, and whether Olig2 plays conserved or pleiotropic roles in neurogenesis of PNS remain to be studied.

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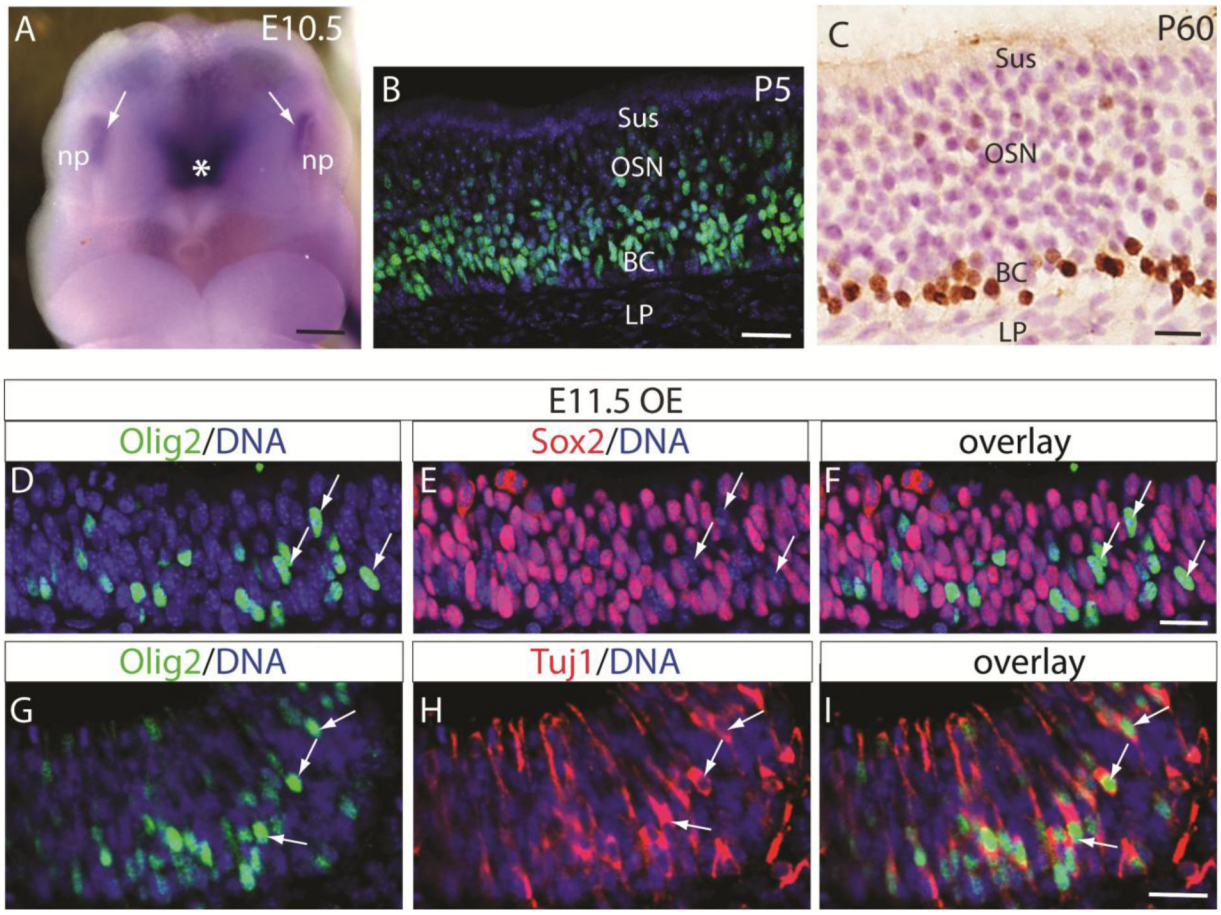

Fig 1. Expression of Olig2 in embryonic, postnatal, and adult olfactory epithelia (OE).

(**A**) Wholemount *in situ* hybridization demonstrates *Olig2* mRNA expression (*while arrows*) in the nasal pit (np) as early as E10.5. *Asterisk* indicates the high expression of *Olig2* in the ventral forebrain as expected. (**B**) Immunolabeling of Olig2 positive cells (*green*, counterstained with DAPI in *blue*) in P5 OE. (**C**) Olig2 positive cells (*brown*, counterstained with hematoxylin and eosin in *purple*) in the adult OE. (**D-F**) Double immunofluorescence of Olig2 and Sox2 in E11.5 OE. Arrows indicate representative Olig2 positive cells that are Sox2 negative. (**G-I**) Double immunofluorescence of Olig2 and Tuj1 in E11.5 OE. Arrows indicate representative Olig2 positive cells with co-immunolabeling of Tuj1. BC, basal cell layer; LP, laminar propria; np, nasal pit; OE, olfactory epithelium; OSN, olfactory sensory neuron layer; Sus, sustentacular cell layer. Scale bars = 200 μm (A), 20 μm (B), 50 μm (C), 20 μm (D-I).

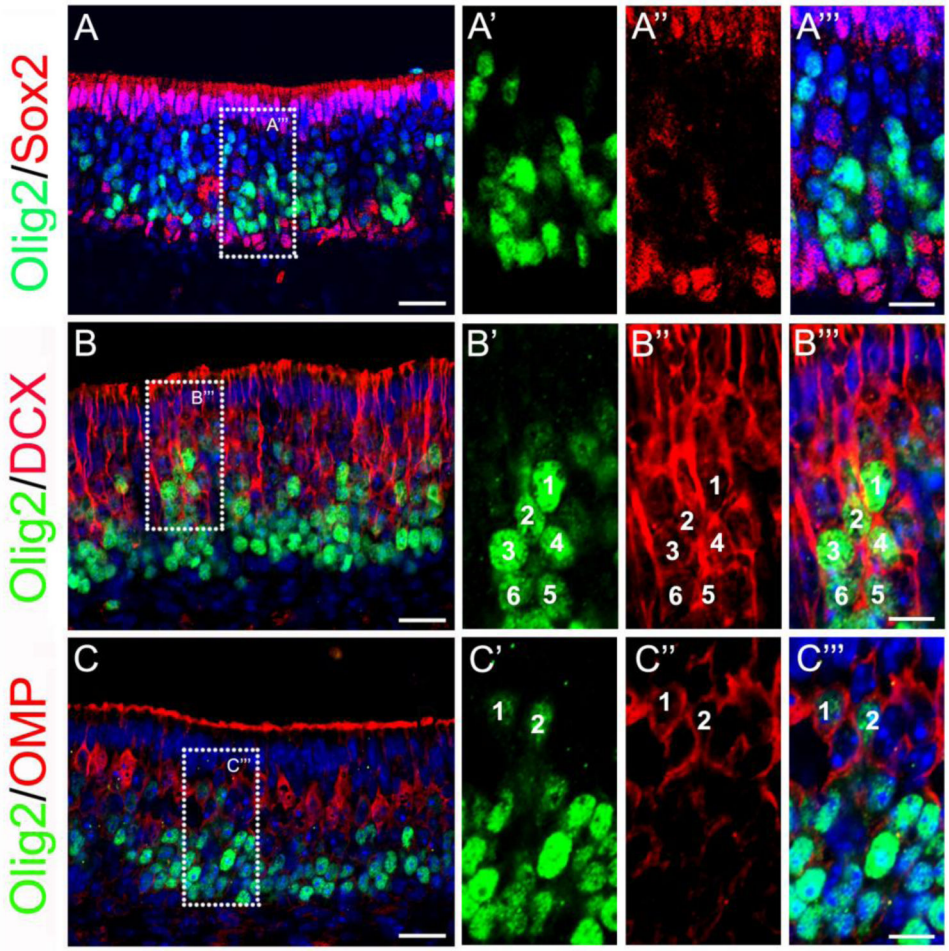

Fig 2. Double immunofluorescence of Olig2 with representative OE lineage markers at E18.5.

(**A-A'"**) At the late embryonic age, Olig2 positive cells (*green*) are closely located atop the Sox2 (*red*) positive basal cell layer and way beneath the Sox2 positive sustentacular cell layer. They do not overlap each other. Dashed rectangle in **A** is enlarged in **A'-A'"** for better resolution. (**B-B'"**) More than one half of the Olig2 positive cells (*green*) are Dcx positive (*red*). Six strongly co-immunolabeled cells are marked in B'-B'". (**C-C'"**) A small portion of Olig2 positive cells are Omp positive (*red*). Two co-immunolabeled cells are marked in C'-C". Nuclei are counterstained by DAPI (*blue*). Scale bars = 50 μm (A,B,C) and 20 μm (A'-C'").

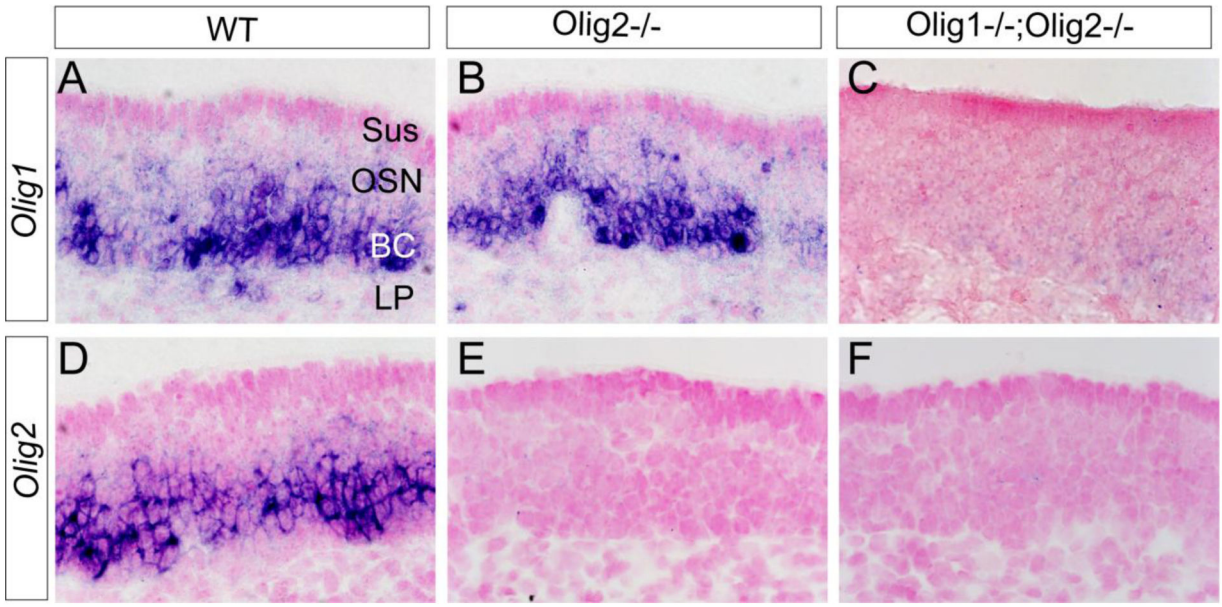

Fig 3. Section *in situ* hybridization for *Olig1/Olig2* mRNAs in the normal and mutant OE at E18.5.

(**A-C**) *Olig1* is also expressed in OE of the *wild-type* (WT) and the single *Olig2-KO* embryos, which is ablated in the *Olig1;Olig2* double KO OE. (**D-F**) *Olig2* expression shows a similar pattern with *Olig1* in the WT, which is ablated in either single *Olig2-KO* or double *Olig1;Olig2* double KO OE. OE sections are counterstained with fast red. BC, basal cell layer; LP, laminar propria; OSN, olfactory sensory neuron layer; Sus, sustentacular cell layer.

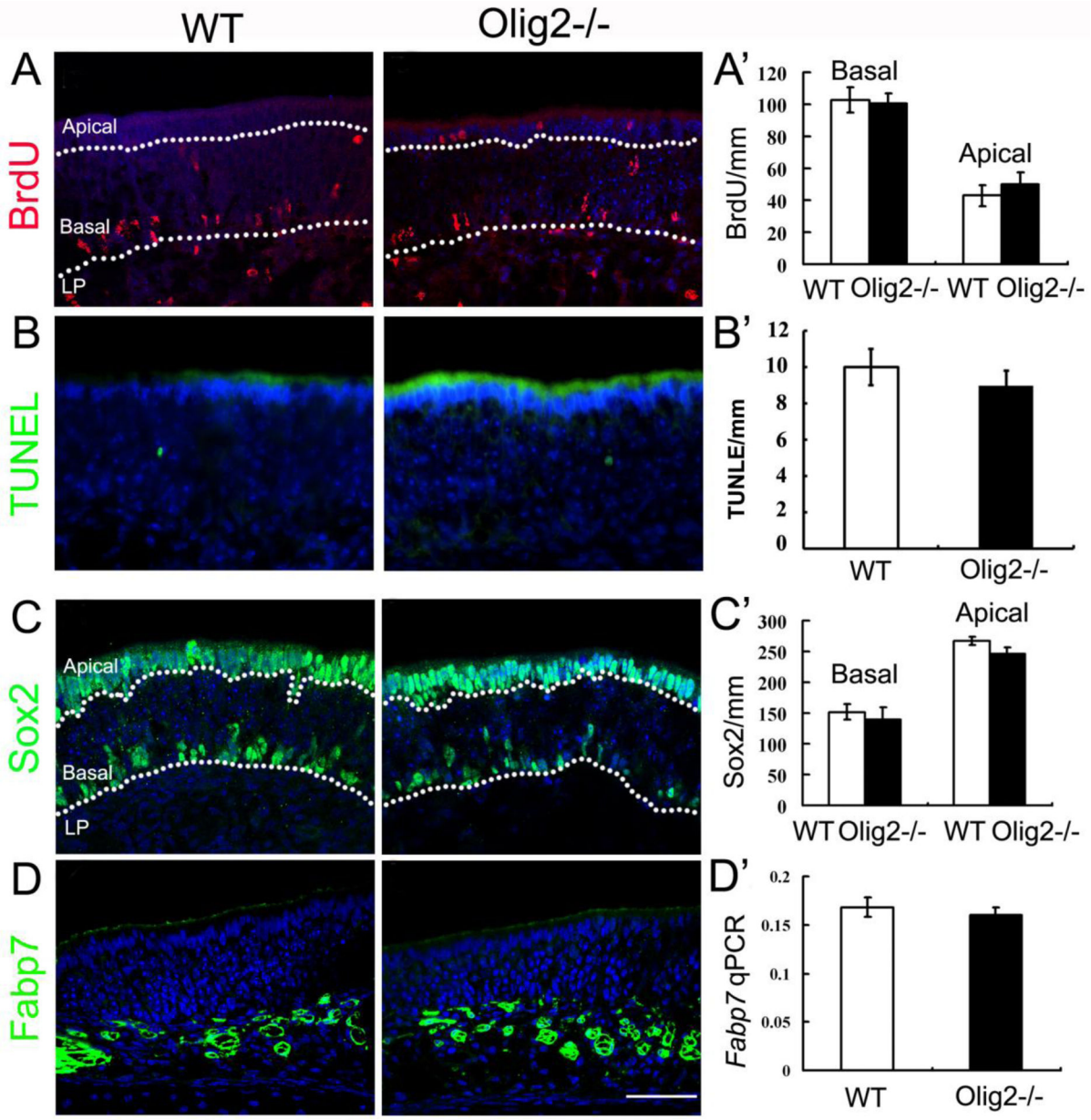

Fig 4. Unchanged proliferation, apoptosis, and gliogenesis in the *Olig2*-deficient OE.

(**A,A'**) BrdU (*red*) immunolabeled OE sections and positive cell numbers in the basal and apical OE of the E18.5 *wild-type* (*WT*) and *Olig2*$^{-/-}$ embryos show no significant changes. N = 3 embryos for each genotype; Student' *t* test. T value: 1.128 (basal) and −0.873 (apical). *P* = 0.463 (basal) and 0.432 (apical). (**B,B'**) No significant changes of TUNEL-labeled apoptotic cells in *Olig2*-deficient OE compared to the *WT* (n = 3 each genotype). Student' *t* test, T value: 0.194, *P* = 0.856. (**C,C'**) Sox2 (*green*) immunolabeled OE sections and positive cell numbers have no significant changes (n = 3 each). Student' *t* test, T value: −0.745 (basal) and 0.955 (apical), *P* = 0.497 (basal) and 0.394 (apical).(**D,D'**) Fabp7 (Blbp) (*green*) immunolabeled OE and qPCR results show no significant changes (n=3 each). Student' *t* test, T value: 0.1178, *P* = 0.304. (G-I). Scale bars = 50 μm.

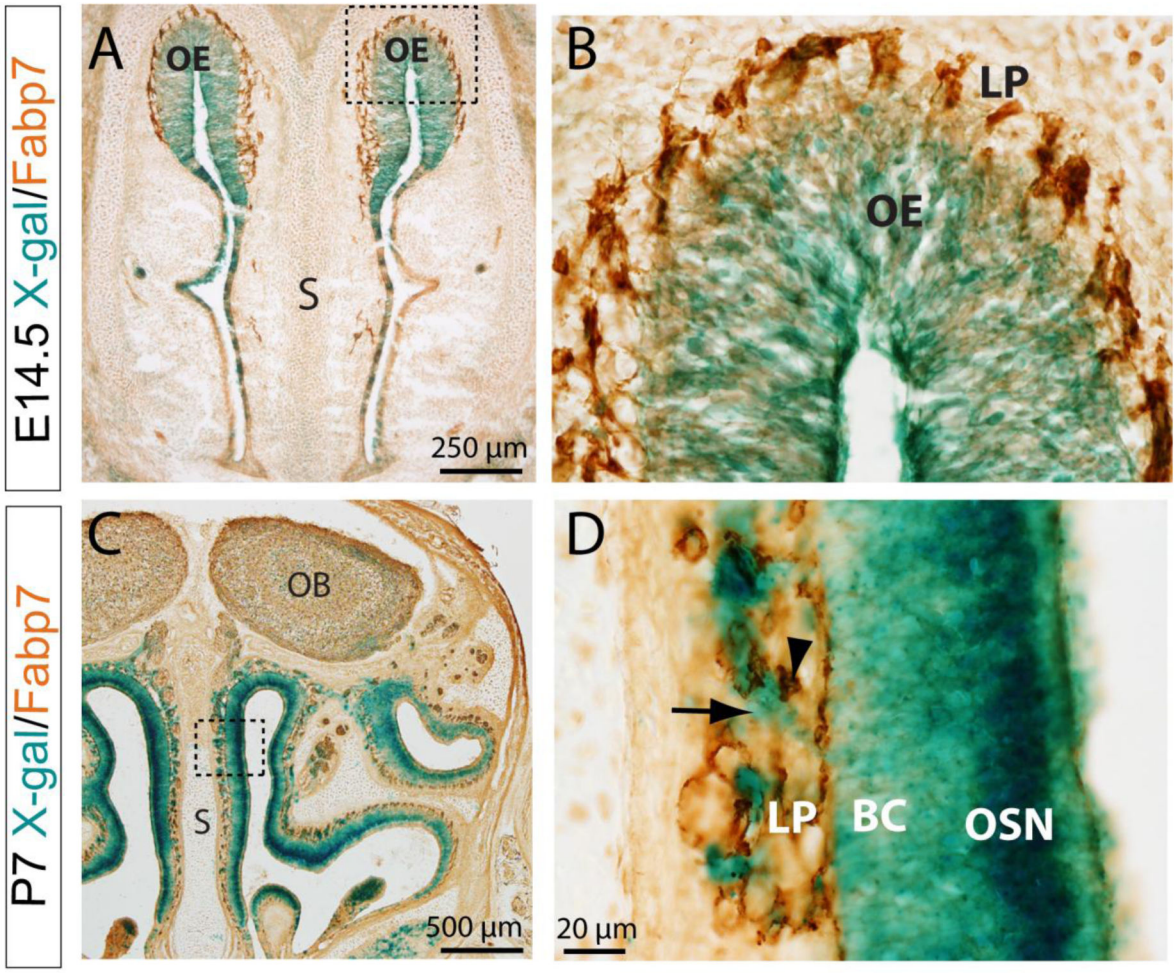

Fig 5. Genetic fate mapping of Olig2 progeny in the peripheral olfactory system.

X-gal staining (*green*) for *Olig2-Cre;Rosa26lacZ* cell lineages and immunolabeling for Fabp7 (Blbp) positive ensheathing cells (*brown*) are shown on the OE sections of E14.5 (**A,B**) and P7 (**C,D**) mice. Panels D & E are enlarged from the dashed squares in A & C, respectively. *Arrowhead* in D indicates a Fabp7 positive ensheathing cell that is adjacent to the Xgal positive axonal fibers (*arrow*). BC, basal cell layer; LP, laminar propria; OE, olfactory epithelium; OB, olfactory bulb; OSN, olfactory sensory neuron layer; S, nasal septum.

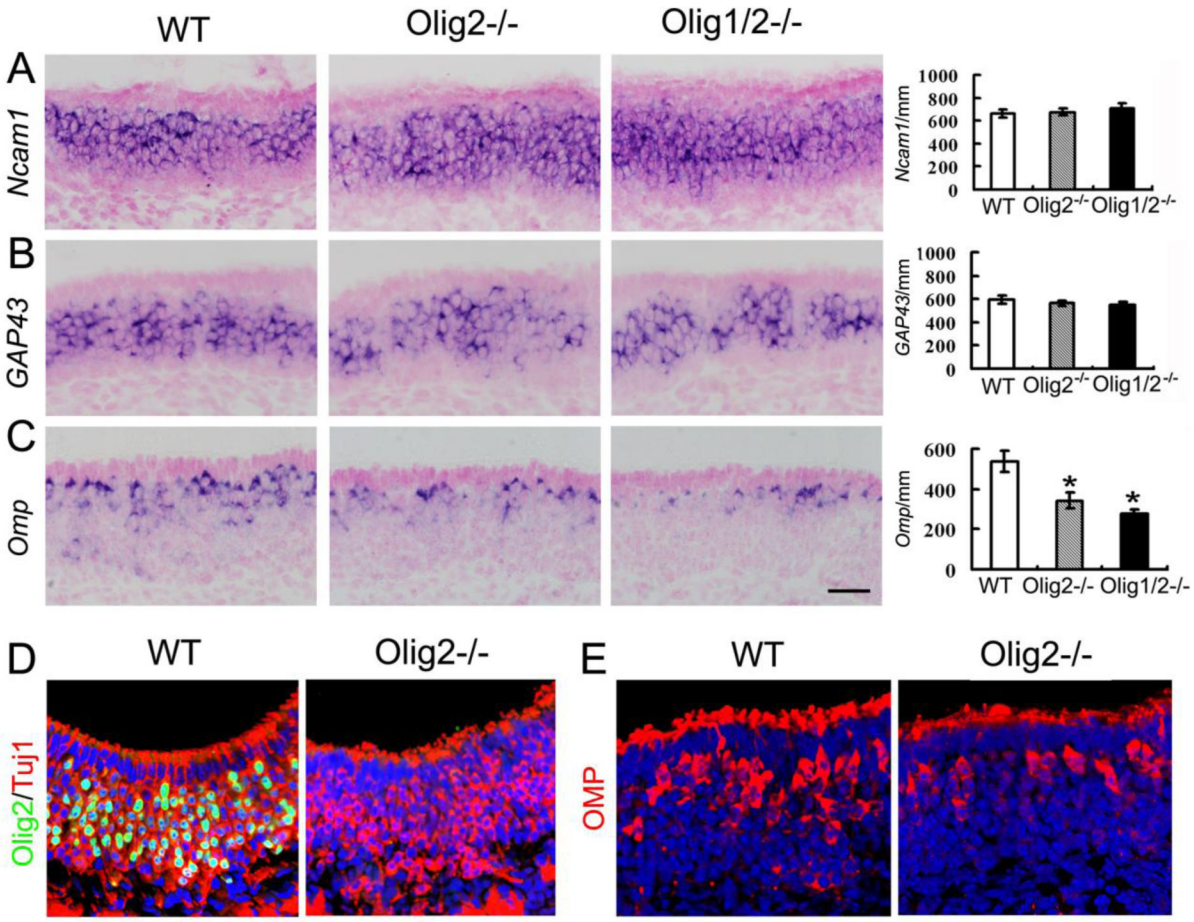

Fig 6. Expression of representative OSN lineage markers in *Olig2* single- and *Olig1;Olig2* double-deficient OE at E18.5.

(**A-C**) *In situ* hybridization and quantification of *Ncam1*, *Gap43*, and *Omp.* Only *Omp*-expressing cells are significantly reduced in the single or double mutants. N = 3 embryos each genotype. One-way ANOVA. For *Ncam1*: F value, 0.206; $P_{Olig2KO \text{ vs } WT} = 0.891$, $P_{Olig1/2KO \text{ vs } WT} = 0.562$. For *Gap43*: F value, 0.038; $P_{Olig2KO \text{ vs } WT} = 0.822$, $P_{Olig1/2KO \text{ vs } WT} = 0.815$. For *Omp*: F value, 11.241; $^{*}P_{Olig2KO \text{ vs } WT} = 0.007$, $^{*}P_{Olig1/2KO \text{ vs } WT} = 0.006$. (**D,E**) Double immunofluorescence of Olig2 with Tuj1 (**D**) and Omp (**E**) demonstrates ablation of Olig2, conserved Tuj1, and consistently reduced Omp positive cells in the *Olig2*-deficient OE.

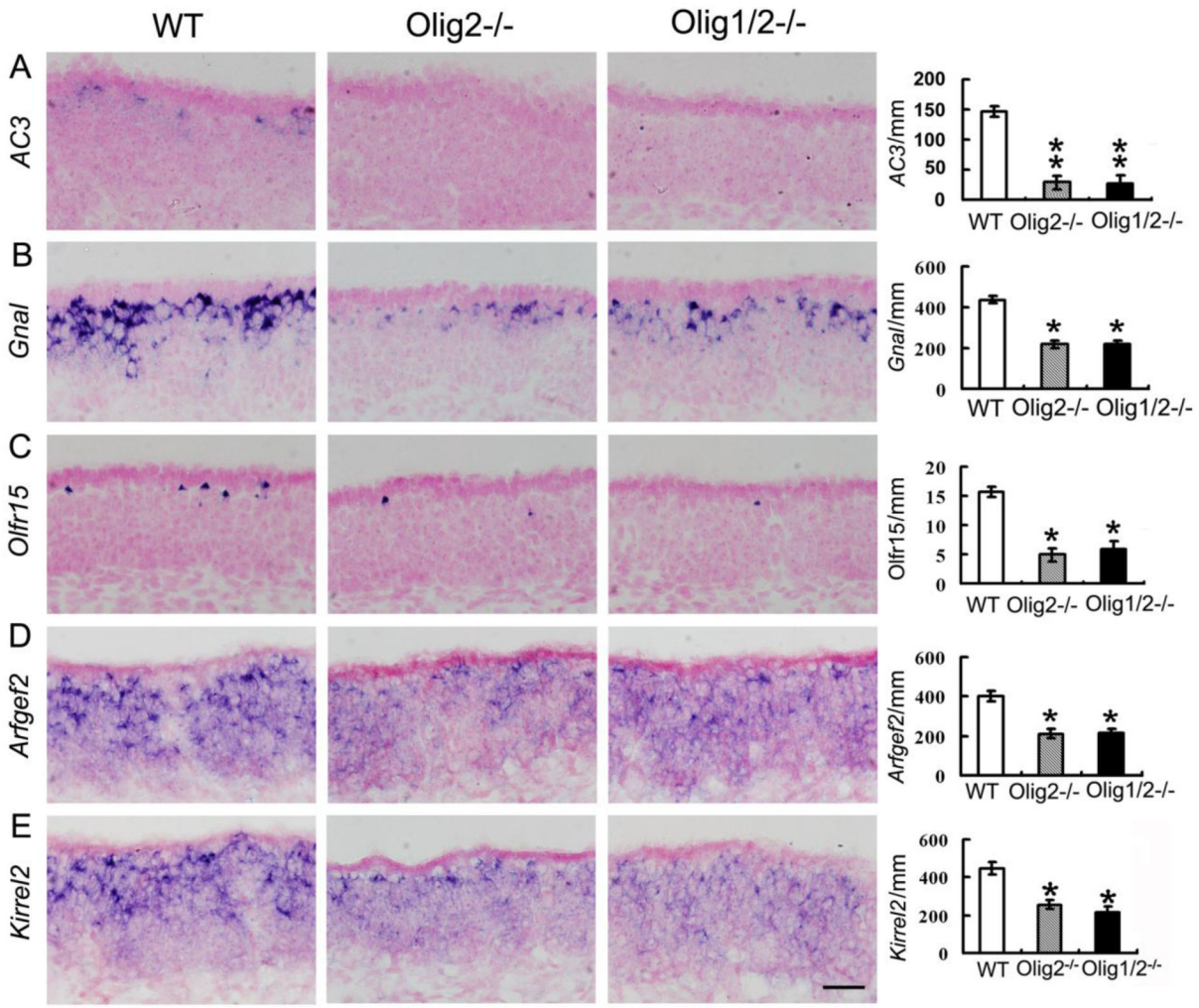

Fig 7. *In situ* hybridization and quantification of representative OSN differentiation marker genes

Acdy3 (*AC3*) (A), *Gnal* (B), *Olfr15* (C), *Arfgef* (D), and *Kirrel2* (E) demonstrate declined expression of these differentiation marker genes in *Olig2* single and *Olig1/Olig2* double knockout OEs at E18.5. Sections from three embryos per genotype were used for each marker. One-way ANOVA. For *Acdy3* (*AC3*): F value, 421.256; $^{**}P_{Olig2KO \text{ vs } WT} < 0.0001$, $^{**}P_{Olig1/2KO \text{ vs } WT} < 0.0001$. For *Gnal*: F value, 16.811; $^{*}P_{Olig2KO \text{ vs } WT} = 0.003$, $^{*}P_{Olig1/2KO \text{ vs } WT} = 0.002$. For *Olfr15*: F value, 24.873; $^{*}P_{Olig2KO \text{ vs } WT} = 0.001$, $^{*}P_{Olig1/2KO \text{ vs } WT} = 0.001$. For *Arfgef*: F value, 25.067; $^{*}P_{Olig2KO \text{ vs } WT} = 0.002$, $^{*}P_{Olig1/2KO \text{ vs } WT} = 0.001$. For *Kirrel2*: F value, 9.865; $^{*}P_{Olig2KO \text{ vs } WT} = 0.005$, $^{*}P_{Olig1/2KO \text{ vs } WT} = 0.029$.

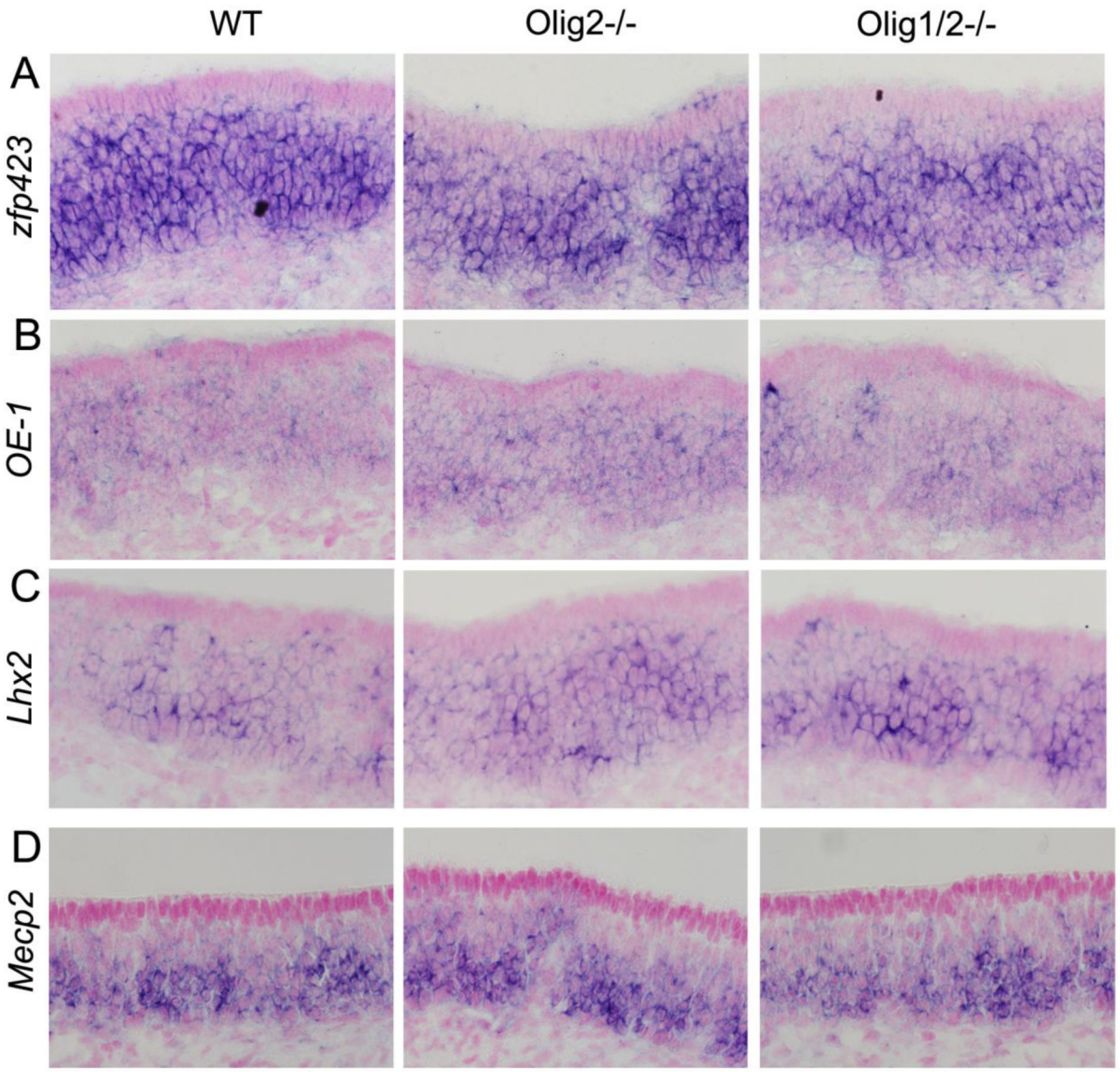

Fig 8. Unchanged expression of transcription factors associated with the terminal differentiation of OSNs in *Olig2* single and *Olig1/Olig2* double knockout OEs at E18.5.

In situ hybridization of *Zfp423, Ebf1* (*OE-1*), *Lhx2*, and *Mecp2* were examined on sections from three embryos per genotype.

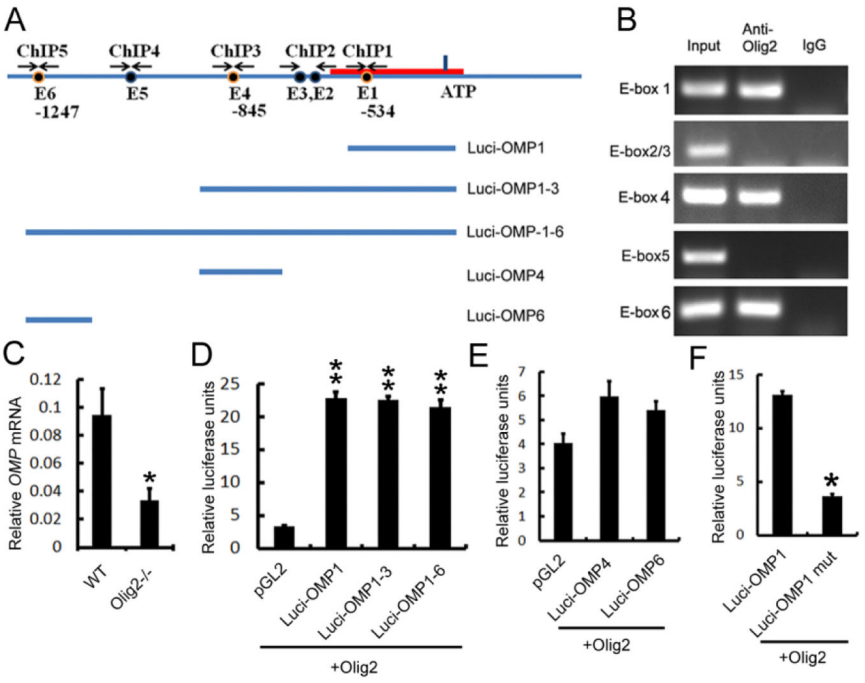

Fig 9. Olig2 binds to E-box and regulates *Omp* promoter activity.

(**A**) Illustration of E-boxes in presumptive *Omp* promoter region, and ChIP and luciferase assay strategies. *Red line* represents the core promoter region of *Omp.* E1-6 stand for the E-boxes 1-6. ChIP1-5 show the PCR amplification regions containing different E-boxes in ChIP assays. *Luci-Omp1~6* represent *Omp* luciferase constructions containing one, three, or all six E-boxes. (**B**) ChIP results demonstrate that Olig2 binds to the E-boxes 1, 4, 6, but not 2/3 and 5 of the *Omp* promoter. (**C**) Real time PCR results confirm the significantly diminished expression of *Omp* in *Olig2*-deficient OE. N = 3 embryos for each genotype, Student' *t* test, T value: 3.219, $P = 0.032$. (**D**) Luciferase assay results demonstrate that Olig2 promotes the transcription of three *Omp* promoter constructs that all contain E-box 1. N = 3 batches of cells. One-way ANOVA. F value, 161.268; $^{**}P_{Luci\text{-}Omp1\ vs\ pGL} < 0.0001$, $^{**}P_{Luci\text{-}Omp1\text{-}3\ vs\ pGL} < 0.0001$, $^{*}P_{Luci\text{-}Omp1\text{-}6\ vs\ pGL} < 0.0001$ (**E**) Constructs which contain E-box 4 or 6 have no significant effects on the expression of *Omp* promoter luciferase activities. N =3 batches of cells. One-way ANOVA. F value, 2.773; $P_{Luci\text{-}Omp4\ vs\ pGL} = 0.099$, $P_{\text{Luci-Omp6 vs pGL}} = 0.079$. (**F**) Mutation in E-box1 significantly abolished the effects of Olig2 on the *Omp* promoter luciferase activity. N = 3 batches of cells. Student' *t* test. T value: 9.633. $^{*}P = 0.001$.