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Anosmin-1a is required for fasciculation and terminal targeting of olfactory sensory neuron axons in the zebrafish olfactory system

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ABSTRACT

The *KAL-1* gene underlies the X-linked form of Kallmann syndrome (KS), a neurological disorder that impairs the development of the olfactory and GnRH systems. *KAL-1* encodes anosmin-1, a cell matrix protein that shows cell adhesion, neurite outgrowth, and axon-guidance and -branching activities. We used zebrafish embryos as model to better understand the role of this protein during olfactory system (OS) development. First, we detected the protein in olfactory sensory neurons from 22 hours post-fertilization (hpf) onward, *i.e.* prior their pioneer axons reached presumptive olfactory bulbs (OBs). We found that anosmin-1a depletion impaired the fasciculation of olfactory axons and their terminal targeting within OBs. Last, we showed that *kal1a* inactivation induced a severe decrease in the number of GABAergic and dopaminergic OB neurons. Though the phenotypes induced following anosmin-1a depletion in zebrafish embryos did not match precisely the defects observed in KS patients, our results provide the first demonstration of a direct requirement for anosmin-1 in OS development in vertebrates and stress the role of OB innervation on OB neuron differentiation.

INTRODUCTION

Genetic neurological diseases offer a powerful approach to identify proteins involved in setting neural networks up and thus, to better understand the molecular processes underlying axon growth and guidance, and neuron migration. Kallmann syndrome (KS) is a human genetic disease that affects OB differentiation, olfactory axon elongation and migration of GnRH (gonadotropin releasing hormone) synthesizing neurons to the brain. As a consequence, the disease is characterised by the association of anosmia (a lack of the sense of smell) (de Morsier, 1954), with hypogonadism resulting from GnRH deficiency (Naftolin *et al.*, 1971).

Links between olfaction and reproductive function have long been reported (Witthen, 1956). In vertebrates, GnRH-synthesizing neurons migrate from the olfactory placode to the hypothalamus, along an olfactory epithelium-forebrain axis of nerve fibres (Schwanzel-Fukuda *et al.*, 1992; Livne *et al.*, 1993). These neurons first travel along olfactory nerves, then penetrate the forebrain just caudal to developing OBs, and ultimately migrate tangentially to reach the hypothalamic area where GnRH secretion takes place (Schwanzel-Fukuda *et al.*, 1988; Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.*, 1989; Norgren and Brackenbury, 1993). Examination of a 19-week-old human foetus carrying a deletion uncovering the *KAL-1* gene showed that both olfactory nerve terminals and GnRH-synthesizing neurons did not enter the brain and had accumulated in the upper nasal region (Schwanzel-Fukuda *et al.*, 1989). This observation led to the hypothesis that the olfactory axon elongation defect is the causative event leading to anosmia and hypogonadism, the OB agenesis being the mere consequence of the lack of contact between OSNs and the presumptive OBs, which in turn prevents their differentiation.

The gene underlying the X chromosome-linked form of Kallmann syndrome (*KAL-1*) was identified several years ago (Franco *et al.* 1991; Legouis *et al.* 1991). The *KAL-1* gene encodes anosmin-1, an extracellular matrix (ECM) protein that displays a modular structure and comprises an amino-terminal whey acidic protein (WAP) motif found in several proteins showing protease inhibitor activity, four contiguous fibronectin type III (FNIII) repeats, which are indicative of extracellular proteins, and a carboxy-terminal histidine-rich region of unknown function (del Castillo *et al.*, 1992). Using *in vitro* approaches, we previously demonstrated that anosmin-1 displays neurite outgrowth, axon guidance and axon branch promoting activities (Soussi-Yanicostas *et al.*, 1996; 1998; 2002). Last, we, and other, recently identified anosmin-1 as a partner of FGFR1 in OB mitral cells in rodents (Gonzalez-Martinez *et al.*, 2005; Ayari and Soussi-Yanicostas, 2007) and a likely cofactor of the

signalling pathway mediated by the chemokine-like SDF1a and its two receptors CXCR4b and CXCR7 in zebrafish embryos (Yanicostas *et al.*, 2008).

Olfactory sensory neurons (OSNs) differentiate within the olfactory placode, a thickened ectoderm that later gives rise to the olfactory epithelium (OE). A previous fate map study has revealed that a large cellular field located along the lateral edge of the anterior neural plate converges through cell movements to form the olfactory placode in zebrafish (Whitlock and Westerfield, 2000). At 20 hpf OSNs extend pioneer axons toward the developing OB. Indeed, OB innervation is accomplished with exquisite precision through sophisticated processes comprising axon guidance and synapse formation. These processes can be divided into at least three steps; First, pioneer OSN axons exit the OE and coalesce to form fascicles that grow toward the presumptive OBs at the rostral tip of the telencephalon. Second, upon reaching the OBs, OSN axons defasciculate tangentially and sort out into smaller subsets toward restricted domains of the OB. Third, the olfactory axons make synaptic connections in target glomeruli with the dendrites of OB output neurons, the mitral cells, and interneurons.

Two *KAL-1* orthologs, namely *kal1a* and *kal1b*, encoding anosmin-1a and anosmin-1b, respectively, have been identified in zebrafish, (Ardouin *et al.*, 2000).

In the present work, we demonstrated that anosmin-1a is accumulated in OSNs and along their axons from 22 hpf onward, *i.e.* prior pioneer axons enter OBs. We also found that fasciculation of OSN axons and their targeting within OB were severely compromised following anosmin-1a depletion. Last, we showed that *kal1a* inactivation also impairs OB development, and differentiation of GABAergic and dopaminergic OB neurons.

MATERIALS AND METHODS

Zebrafish strains

Zebrafish (*Danio rerio*) were maintained as described by Westerfield (1995). Embryos were produced in our facility using standard conditions. Wild-type embryos were from the AB strain. Animals from the *omp-Tau::GFP* transgenic line were kindly provided by M. Mishina (Yoshida *et al.*, 2002).

Antibody production and fluorescent immunocytochemistry on whole mount embryos

Antibodies raised against zebrafish anosmin-1a (Genbank AF163310) have been previously described (Ernest *et al.*, 2007; Yanicostas *et al.*, 2008). Anti-anosmin-1a, anti-Calretinin (CliniSciences) and anti-Tyrosine Hydroxylase (TH) (Boehringer) antibodies were used at 1:1000, 1:500 and 1:500 dilutions, respectively. Immunodetections of anosmin-1a, Calretinin and Tyrosine Hydroxylase (TH) were performed as described in Yanicostas *et al.* (2008).

Morpholino-mediated gene inactivation

All morpholino-oligonucleotides (MO) used in this study have been previously described, *i.e.* *kal1a*-specific morpholino, MO *kal1a*, the corresponding mismatching control morpholino, mmMO *kal1a*, and *kal1b*-specific morpholino: MO *kal1b*, which was also used as control, (Yanicostas *et al.*, 2008). Throughout this study, complete or nearly complete anosmin-1a depletion was achieved following injection of solutions containing 0.5 mM MO *kal1a*.

*Production of MO *kal1a*-insensitive *kal1a* transcripts and phenotypic rescue*

MO *kal1a*-insensitive *kal1a* transcripts were synthesised as previously described (Yanicostas *et al.*, 2008). For rescue experiments, a mix containing MO *kal1a* (0,5 mM) and MO *kal1a*-insensitive *kal1a* mRNA (1 μ M), was injected into embryos at the one-cell stage according to standard protocols.

Whole-mount in situ hybridization

In situ hybridization experiments were performed using the R&D Systems kit (R&D Systems Europe, Lille, France) as previously described (Yanicostas *et al.*, 2008). The following cDNA were used as templates to synthesize RNA probes: *kal1a* (Ardouin *et al.*, 2000; Genbank AF163310), and *GAD67* (Martin *et al.*, 1998; Genbank CR 384078)

Microscopy, Confocal microscopy and ApoTome microscopy

Fluorescent microscopy images were acquired on a Zeiss LSM confocal microscope. *In vivo* imaging of olfactory system development in *OMP-Tau::GFP* transgenic embryos was recorded using an epifluorescent AXIO imager Z1 microscope (Zeiss) equipped with an ApoTome system (Zeiss). Prior to imaging, live embryos were anaesthetized in tricaine dissolved in fish water for 5 minutes and then mounted in a drop of low-melting NuSieve GTG agarose (FMC BioProducts) cooled at 38°C.

Quantification of Tyrosine Hydroxylase immunolabelling and statistical analysis

To quantify Tyrosine Hydroxylase immunolabelling the mean grey-scale intensity was measured using the ImageJ software (NIH, Bethesda, MD) on randomly selected images of wild-type embryos (n=10) and *kal1a* morphants (n=10). Immunolabelling intensities were statistically analysed using a Student's t-test and $P < 0.05$ was considered statistically significant.

RESULTS

Previous studies demonstrated that though the *kal1a* gene played an essential role in several developmental processes in zebrafish embryos, including GnRH neuron migration (Whitlock *et al.*, 2005), the journey of the posterior lateral line primordium (Yanicostas *et al.*, 2008) and OS development (the present study), none of these processes were defective following anosmin-1b depletion (Whitlock *et al.*, 2005; Yanicostas *et al.*, 2008; data not shown). Therefore, we focused our investigations on the function of anosmin-1a in OS development.

Distribution of anosmin-1a in the olfactory system

We first investigated the distribution of anosmin-1a in the developing OS of zebrafish embryos aged from 20 hpf to 120 hpf using two polyclonal anti-anosmin-1a antibodies, which have been previously described (Ernest *et al.*, 2007; Yanicostas *et al.*, 2008).

In the developing OS, anosmin-1a was detected from 22 hpf onward in OSNs, at around the stage their pioneer axons exit the olfactory epithelium (data not shown). At 36 hpf, though anosmin-1a accumulation persisted in OSNs, the protein was also observed in, or around, growing pioneer OSN axons extending toward presumptive OBs (Fig. 1A). At this stage, anosmin-1a-immunoreactive OSN axons were also observed within OBs. At 72 hpf, the same pattern was observed, with anosmin-1a being detected in olfactory neurons and along their axons (Fig. 1B-C). Thus, anosmin-1a is expressed in OSNs and their axons throughout OS development.

In presumptive OBs, no immuno-reactive cells were detected between 22 and 48 hpf (data not shown). During OB differentiation, cells accumulating anosmin-1a were only detected from 72 hpf onward (Fig.1D, and data not shown).

Anosmin-1a is required for OE differentiation and proper fasciculation of OSN axons

We made use of morpholino oligonucleotide (MO)-mediated gene inactivation to investigate the consequences of anosmin-1a depletion on OS development. To visualize OSNs and their axons, we used embryos of the *omp-tau::GFP* transgenic line as recipients for MO *kal1a* injection. In this line, embryos display an intense fluorescent labelling of OSNs and their axons (Yoshida *et al.*, 2002). As previously described, analysis of 24 hpf *omp-tau::GFP* embryos showed tens of fluorescently

labelled OSNs. These neurons were arrayed in a circle and extended their axons toward presumptive OBs (Fig. 2A). After exiting the OE, OSN axons coalesced to form tightly fasciculated nerve fibres. At the same stage, we observed several defects in the OS of *omp-tau::GFP kal1a* morphants (n=38) (Fig. 2B-E). First, we detected a severe disorganization of the olfactory placode that comprised irregularly deposited OSNs. The growth of pioneer olfactory axons was also markedly impaired and the few shortened axons that exited OE did not fasciculate properly and appeared to elongate at random, sometime coalescing onto small fascicule-like structures. At 48 hpf, GFP staining of *omp-tau::GFP* embryos revealed that the nerve fibres comprising OSN axons had crossed the boundary between the olfactory placode and the OB (BPB) through a discrete region, as described by Hansen and Zeiske (1993). After crossing the BPB, OSN axons defasciculated to form thinner axon fascicules that projected toward distinct OB regions (Fig. 2F). At the same stage, in *kal1a* morphants (n=36), the overall organization of the olfactory epithelium and the regular circular distribution of OSNs were compromised, with several OSNs wandering outside the OE (Fig. 2J). In addition, though olfactory axons extended properly toward the rostral tip of the telencephalon, they formed dishevelled bunches of axon fibres instead of coalescing onto tightly fasciculated nerve fibres (Fig. 2G-J). At 72 hpf, analysis of *omp-tau::GFP* embryos showed that within OBs the olfactory nerves fully defasciculated (Fig. 2K). At the same stage, *omp-tau::GFP kal1a* morphants (n=36) showed several defects. First, the regular circular distribution of the OSNs was markedly disorganized in almost all anosmin-1a-depleted embryos (33/36) (Fig. 2L-O) and, in the most affected morphants (5/36), the OE was partially split into two adjacent and poorly organized structures (Fig. 2M). The olfactory tract comprising OSN axons also displayed an abnormal organization. These axons appeared fully dishevelled (Fig. 2L-O), sometime coalescing onto two (Fig. 2L) or more fascicules (Fig. 2N). Last, though most OSN axons entered OBs, their subsequent targeting also was compromised, as illustrated by the disorganized axonal network observed in OBs (Fig. 2L-O).

To verify that the defects observed in *kal1a* morphants were not due to a non-specific effect of the morpholino used in this study, we tested whether co-injection of a MO *kal1a*-insensitive *kal1a* RNA was able to rescue the defects observed in MO *kal1a*-injected embryos (Fig. 3C). The results show that in almost all (42/45) embryos co-injected with MO *kal1a* (0.5 mM) and MO *kal1a*-insensitive *kal1a* RNA (1 μ M), OE differentiation, fasciculation of OSN axons, and OB innervation were not significantly different to that observed in wild-type embryos (Fig. 3D), or those

injected with a mis-matching *kal1a* morpholino (n=52), mmMO *kal1a* (Fig. 3A) or the MO *kal1a*-insensitive *kal1a* RNA alone (n=38) (Fig. 3B).

Kal1a inactivation impairs innervation of OB neurons by OSN axons

Analysis of *omp-tau::GFP kal1a* morphants suggested that anosmin-1a depletion impaired both the fasciculation of OSN axons and proper establishment of synaptic connections between OSN axons and the dendrites of OB neurons. Calretinin is accumulated to high levels in OSNs and along their axons (Castro *et al.*, 2006), making this protein a good marker to visualize these axons and their behaviour within presumptive OBs and, thus, the effect of anosmin-1a depletion on innervation of presumptive OBs. In 72 hpf wild-type embryos (n=9), Calretinin accumulation was detected in OSNs and in, or around, the fibre that comprises OSN axons and connects the OE to presumptive OBs (Fig. 4A). Within OBs, Calretinin-positive axons mainly projected toward two discrete proto-glomeruli (Fig. 4A). In *kal1a* morphants (n=13), Calretinin staining confirmed that OSN axons misrouted within OBs, some of them projecting toward single and irregularly shaped proto-glomeruli (Fig. 4B). In 120 hpf wild-type embryos (n=9), the same pattern of Calretinin accumulation was observed in OBs with OSN axons projecting toward glomeruli (Fig. 4C). At the same stage, in anosmin-1a depleted embryos (n=13), we found a marked disorganization of Calretinin immuno-reactive axons, which appeared to project at random, combined with a lack of properly differentiated OB glomeruli (Fig. 4D).

Kal1a inactivation compromises the differentiation of several populations of OB neurons

While the examination of a 19-week-old human foetus carrying a deletion of the *KAL-1* gene strongly suggested an essential requirement for anosmin-1 in terminal elongation of OSN axons toward OB anlagen (Schwandzel-Fukuda *et al.*, 1989), in zebrafish embryos, anosmin-1a depletion does not fully block the elongation of OSN axons toward OBs. Furthermore, because the early arrest of OSN axons was proposed to be the causative defect leading to OB agenesis in X-linked KS, we next investigated whether OB development and/or differentiation were compromised following *kal1a* inactivation.

Visual examination of the telencephalon morphology in 120 hpf wild-type embryos (n=8), *kal1a* morphants (n=12), and embryos injected with a control MO

(n=9), first revealed that OBs displayed an abnormal shape in *kal-1a* morphants (Fig. 5B), when compared to those of wild-type embryos (Fig. 5A) or embryos injected with a control MO (Fig. 5C). GABAergic neurons express glutamate decarboxylase 67, which is encoded by the *GAD67* gene (Martin *et al.*, 1998). We therefore investigated by *in situ* hybridization on whole-mount dissected brain of 120 hpf embryos using a *GAD67* probe, whether anosmin-1a depletion affected the differentiation of GABAergic OB neurons. In wild-type embryos, OBs comprised a large population of *GAD67* expressing cells located within the internal cell layer (Fig. 5A, C). In contrast, in *kal1a* morphants, no *GAD67* positive cells were detected (Fig. 5B), demonstrating that anosmin-1a depletion prevented the differentiation of GABAergic OB neurons. To further investigate the requirement for anosmin-1a in OB differentiation, we tested whether the differentiation of dopaminergic OB neurons was also impaired following MO-mediated *kal1a* inactivation. We visualized these interneurons by immunocytochemistry using an antibody raised against Tyrosine Hydroxylase (TH), a rate-limiting enzyme in dopamine synthesis. In OBs of wild-type 120 hpf embryos (n=11), we observed a large population of TH expressing cells in the glomerular cell layer (Fig. 6A).

In OBs of 120 hpf *kal1a* morphants (n=13), quantification revealed a significant decrease (by ~ 50%) of TH labelling intensity when compared to that observed in wild-type embryos (wild-type embryos, 109.8 ± 20.04 , n=10; *kal1a* morphants: 55.68 ± 20.24 , n=10; $p < 0.01$) (Fig. 6B), thus showing that anosmin-1a depletion also severely compromised differentiation of dopaminergic OB neurons.

DISCUSSION

Species-specific tissue distribution of anosmin-1/anosmin-1a in the OS

We observed a strong accumulation of anosmin-1a in OSNs and along their pioneer axons in zebrafish embryos aged from 22 hpf onward. A similar pattern of accumulation was seen at 48 and 72 hpf, except that immuno-reactive OSN axons were also detected within the presumptive OBs. By contrast, accumulation of anosmin-1a in the presumptive OBs was detected from 72 hpf onward, approximately two days after the earliest pioneer OSN axons enter the brain. These results are consistent with previous *in situ* analyses of the pattern of expression of *kal1a* transcripts, which were faintly detected in presumptive OBs at 37 hpf, and transcribed to high levels in these structures from 72 hpf onward (Ardouin et al., 2000).

The distribution of anosmin-1a in the brain of zebrafish embryos did not match precisely with earlier investigations of the distribution of *KAL-1* mRNA and/or anosmin-1 in chick, human, rat and musk shrew embryos (Legouis et al., 1993; 1994; Rugarli et al., 1993; Soussi-Yanicostas et al., 1996; Hardelin et al., 1999; Dellovade et al., 2003; Clemente et al., 2008). In particular, investigations performed on human foetuses aged from 4 to 10 weeks of gestation demonstrated a strong accumulation of the protein in OB from 5 week of gestation onward and a complete absence of anosmin-1 expressing cells in the OE (Hardelin et al., 1999). These data suggested that anosmin-1 plays an essential permissive, or instructive, role in the targeting of olfactory axons toward the OBs (Hardelin et al., 1999). In the present study, though we detected anosmin-1a accumulation in OSNs from 22 hpf onward, prior their pioneer axons reach presumptive OBs, we did not detect accumulation of the protein in OB anlagen at this stage. These data suggest that anosmin-1a does not play a permissive, or instructive, role for proper targeting of OSN axons in zebrafish embryos. Moreover, following anosmin-1a depletion, OSN axons projected properly toward the rostral region of the telencephalon and eventually reached OBs. These data contrast with the examination of a 19-week-old human foetus carrying a chromosomal deletion encompassing the *KAL-1* gene that showed an accumulation of olfactory axons in the upper nasal region (Schwandzel-Fukuda et al., 1989).

Taken together, these data suggest that though anosmin-1 is accumulated in the developing OS in humans and fish and plays an essential role for proper

innervation of OBs in both species, this protein appears to play distinct and species-specific function in this process.

Anosmin-1a, a factor required for the fasciculation and terminal targeting of OSN axons

In vertebrates, individual OSNs express only one odorant receptor (OR) among ~1000 genes in rodents and ~100 genes in fish (Mombaerts, 1999). OSNs expressing a given OR are scattered within the OE but they project their axons onto specific OB glomeruli to create a neural representation of odorant stimuli, an odor map in mice (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaert *et al.*, 1996). Though, genetic manipulation of specific OR genes in mice have shown that the ORs themselves play an instructive role in glomerular targeting of OSN axons, other molecules involved in the guidance of olfactory axons have been identified (St John *et al.*, 2002). Ablation experiments have demonstrated that pioneer neuron axons play an essential role for proper elongation and guidance of OSN axons suggesting these pioneer axons may serve a guidance function for later developing olfactory axons (Whitlock and Westerfield, 1998). Furthermore, as pioneer axons do not express odorant receptors in zebrafish, Whitlock and Whiterfield (1998) put forward the hypothesis that these receptors are not involved in the guidance of pioneer axons and that other cues, such as cell-surface glycoproteins, guide these axons to the telencephalon. Several molecules have been identified as guidance cues controlling the navigation of OSN axons toward the presumptive OBs. Semaphorin3A and EphrinA have been identified as key factors for proper axon sorting within the olfactory nerve layer (Schwartz *et al.*, 2000; Tanigusi *et al.*, 2003) and termination of OSN axons onto precisely defined glomeruli (Cutforth *et al.*, 2003), respectively, in rodents. Miyasaka *et al.* (2005) identified Robo2/Slits as the components of a signalling pathway that play a key role in the guidance of nascent OSN axons to presumptive OBs in zebrafish embryos. In particular, in zebrafish embryos homozygous for the *robo2* mutation, *astray (ast)*, OSN axons were misrouted and, the spatial arrangement of glomeruli in OB was disorganized in adults (Miyasaka *et al.*, 2005). Moreover, *ast* embryos showed defects in both the fasciculation of OSN axons and proto-glomerular organization in OBs (Miyasaka *et al.*, 2005), which were reminiscent, though not identical, to the phenotypes observed in *kal1a* morphants. Anosmin-1 and anosmin-1a have been shown to be likely co-factors of FGFR1 (Gonzalez-Martinez *et al.*, 2004; Ayari *et al.*, 2007) and CXCR4b (Yanicostas *et al.*, 2008), respectively. Moreover, it has been demonstrated that

anosmin-1 binds to urokinase-type plasminogen activator (Hu *et al.*, 2004) suggesting that the extra-cellular matrix anosmin-1 acts as a scaffold protein that recruits proteases and locally increases the proteolytic degradation of extracellular matrix components, and thus the release or activation of receptor ligands. In this context, an attracting hypothesis would be that anosmin-1/anosmin-1a is a cofactor of signalling pathways controlling the guidance of olfactory axons toward OBs. Consistent with this hypothesis, we have shown that anosmin-1 stimulated neurite outgrowth of OSN axons in rat (Soussi-Yanicostas, unpublished results).

Notwithstanding the role of anosmin-1a in terminal extension of OSN axons within OBs, in *kal1a* morphant embryos, the olfactory epithelium appeared disorganized, with a few isolated OSNs wandering outside the OE. These data, which suggested that anosmin-1a is essential to maintain the cohesion of OSNs, were in agreement with the cell adhesion properties of the protein observed *in vitro* (Soussi-Yanicostas *et al.*, 1996). Similarly, the fasciculation defect of OSN axons and their dishevelled appearance in *kal-1a* morphants reinforced the hypothesis of anosmin-1a acting as an adhesion molecule required for both the cohesion of OSNs within the OE and coalescence of their axons as they exit the olfactory epithelium.

Anosmin-1a is required for the differentiation of several OB neuron populations

Patients suffering X-linked KS display OB agenesis (Schwandzel-Fukuda *et al.*, 1989). Our results show that *kal-1a* inactivation impaired the spatial arrangement of glomeruli within OBs. However, anosmin-1a accumulation in OBs was detected from 72 hpf onward, approximately 2 days after the earliest pioneer OSN axons cross the BPB, suggesting that the absence of differentiated glomeruli in the OBs of *kal1a* morphants likely relied on OSN axon misrouting within OB. Similarly, the nearly complete lack of GABAergic neurons and the marked decrease in the number dopaminergic neurons in anosmin-1a depleted embryos, strongly suggested that proper innervation of OB by OSN axons is crucial for full differentiation of these two neural populations.

Our data provide functional evidences that zebrafish anosmin-1a plays essential functions in OE development, OSN axon fasciculation, and OB innervation and differentiation, suggesting an evolutionarily conserved function for this protein in OS development. However, the distinct accumulation pattern of anosmin-1 in OE in chick and mammals compared to that observed in fish, also suggests that this protein plays species-specific roles in OS development. This hypothesis is strengthened by the presence of OBs, though of abnormal shape, in *kal-1a*

morphants, while KS patients display complete OB atrophy. However, whereas the human foetus studied by Schwandzel-Fukuda *et al.* (1989) was fully devoid of anosmin-1, residual low levels of anosmin-1a might persist in *kal-1a* morphants leading to partial OB differentiation.

Notwithstanding these differences, our results are an important advance in the field, which paves the way to better understand the molecular processes underlying the requirement for anosmin-1a in OS development.

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FIGURE LEGENDS

Figure 1. Accumulation of anosmin-1a in the olfactory system. (A-C) Frontal view with dorsal region to the top; (D) dorsal view with anterior to the left. Whole-mount immunodetection of anosmin-1a in a 36 (A) and 72 hpf (B-D) embryos. Arrows indicate immuno-reactive OSN axons, which have entered olfactory bulbs. Double-headed arrows indicate the dorso-ventral (D-V) and antero-posterior (A-P) axes. OB, olfactory bulb; OE, olfactory epithelium; n, nostril.

Figure 2. Effects of anosmin-1a depletion on the differentiation of the olfactory epithelium and axon pathfinding of olfactory sensory neurons. Frontal views of living *omp-tau::GFP* zebrafish embryos at 24 (A-E), 48 (F-J) and 72 hpf (K-O). Wild-type embryos (A, F, K) and *kal1a* morphants (B-E, G-J, L-O). The same wild-type embryo (A, F, K) and *kal1a* morphants (B, G, L; C, H, M; D, I, N and E, J, O) were visualized at 24, 48, and 72 hpf. Dorsal is to the top, and ventral to the bottom. Large white arrow indicates tightly fasciculated OSN axons, small white arrows show defasciculated axon fibres within olfactory bulbs, red arrows indicate disorganized olfactory sensory axons and small green arrows point to dissociated olfactory sensory neurons. OE, olfactory epithelium; ON, olfactory nerve; OB, olfactory bulbs.

Figure 3. Effects of anosmin-1a depletion can be rescued by ectopic expression of the protein. Frontal views of 48 hpf *omp-tau::GFP* embryos injected with mismatching control *kal1a* MO (A), MO *kal1a*-insensitive *kal1a* RNA (B), MO *kal1a* (C) or *omp-tau::GFP kal1a* morphant co-injected with MO *kal1a*-insensitive *kal1a* RNA (D). Dorsal is to the top, and ventral to the bottom. White arrows indicate defasciculated axon fibres within olfactory bulbs. Red arrows show disorganized olfactory sensory axons. OE, olfactory epithelium; ON, olfactory nerve; OB, olfactory bulbs.

Figure 4. Effects of anosmin-1a depletion on the terminal axon pathfinding of olfactory sensory neurons within olfactory bulbs. Frontal views of calretinin accumulation in the olfactory system of wild-type (A, C) and MO *kal1a*-injected embryos (B, D) at 72 (A, B) and 120 hpf (C, D). White arrows indicate protoglomeruli. Red arrows show disorganized olfactory sensory axons seen within olfactory bulbs. OE, olfactory epithelium; OB, olfactory bulbs.

Figure 5. Anosmin-1a depletion impairs the differentiation of GABAergic olfactory bulb neurons. Whole mount *in situ* detection of *GAD67* transcripts on dissected brains of 120 hpf wild-type embryo (A), MO *kal1a* morphant (B), and embryo injected with a control MO (C). OB, olfactory bulbs.

Figure 6. Anosmin-1a depletion impairs the differentiation of dopaminergic olfactory bulb neurons. Whole mount immunodetection of Tyrosine Hydroxylase (TH) on dissected brains of 120 hpf wild-type embryo (A), and MO *kal1a* morphant (B). OB, olfactory bulbs.

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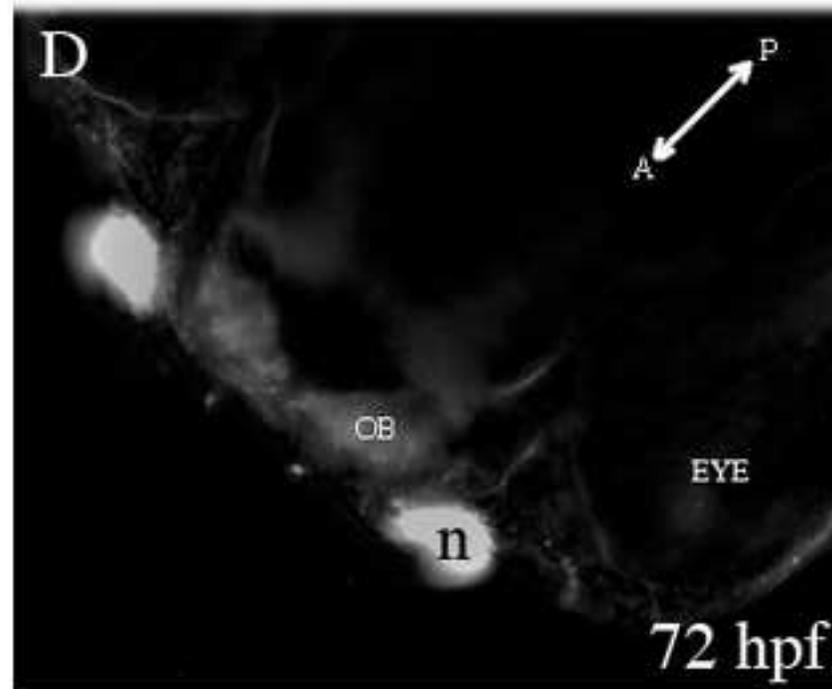
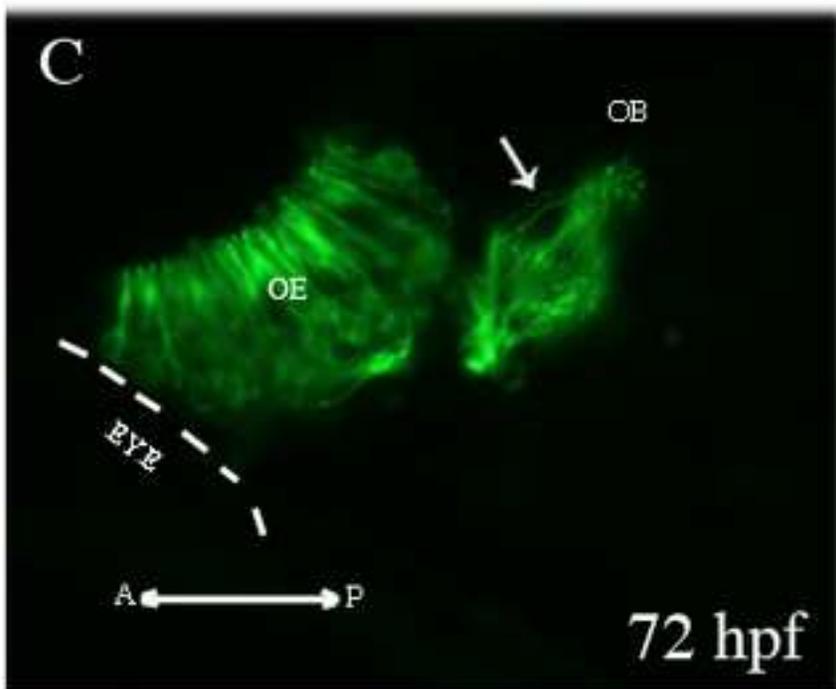
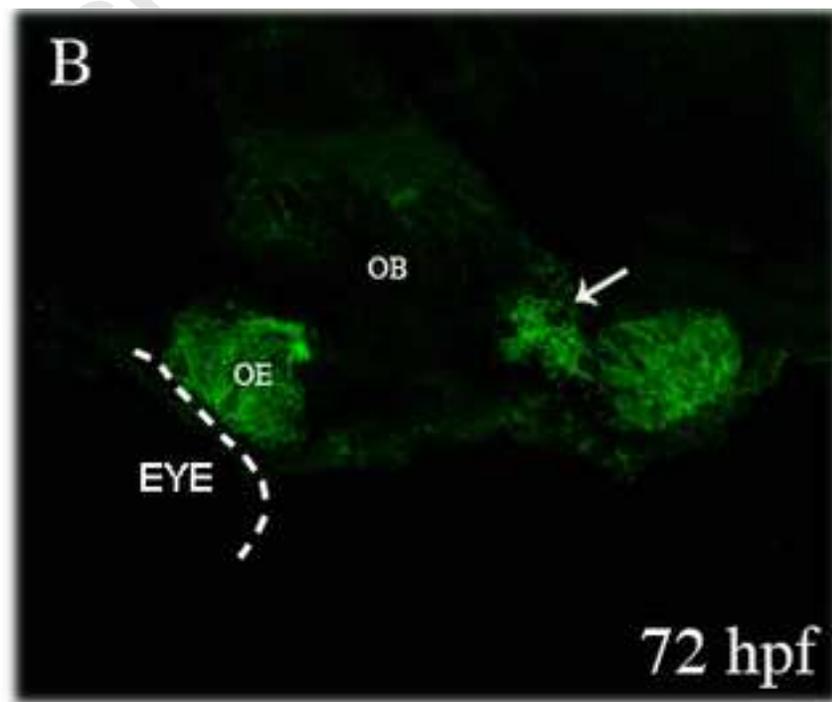
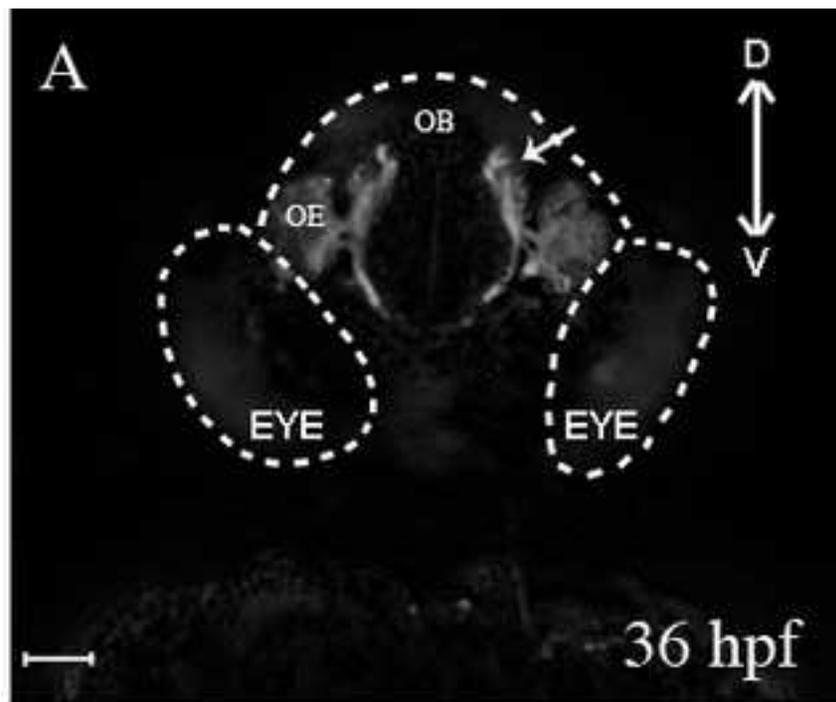
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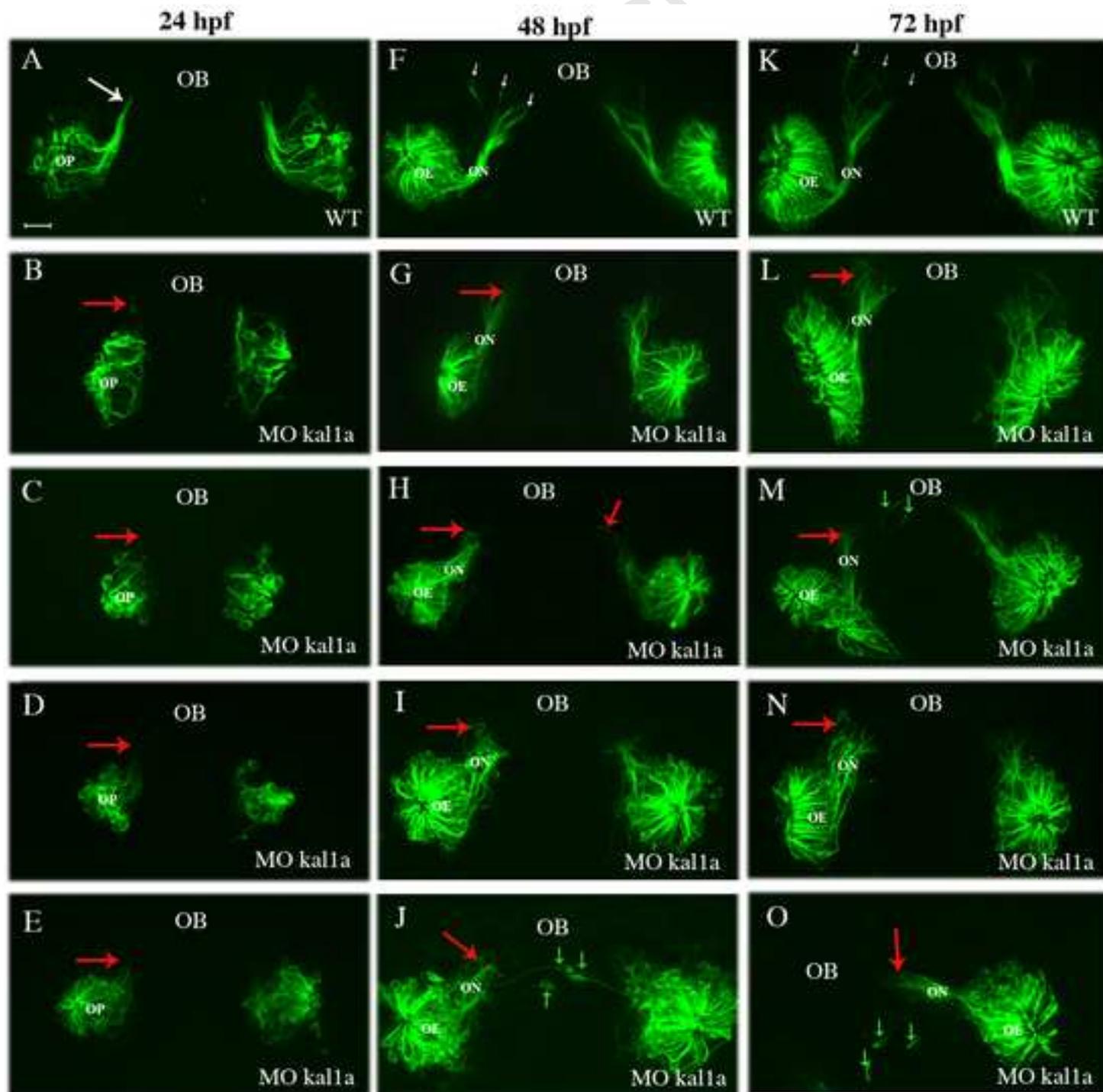
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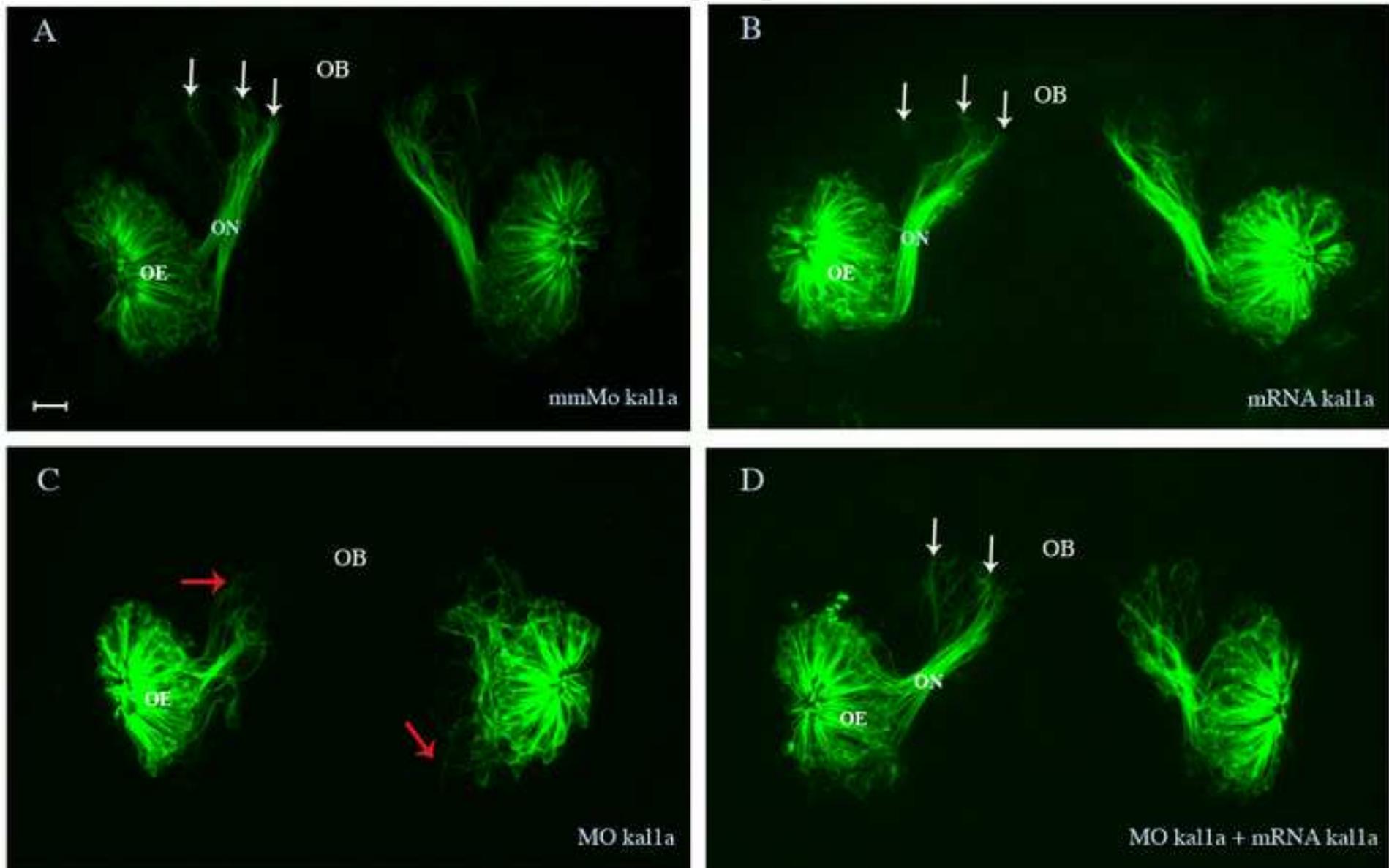
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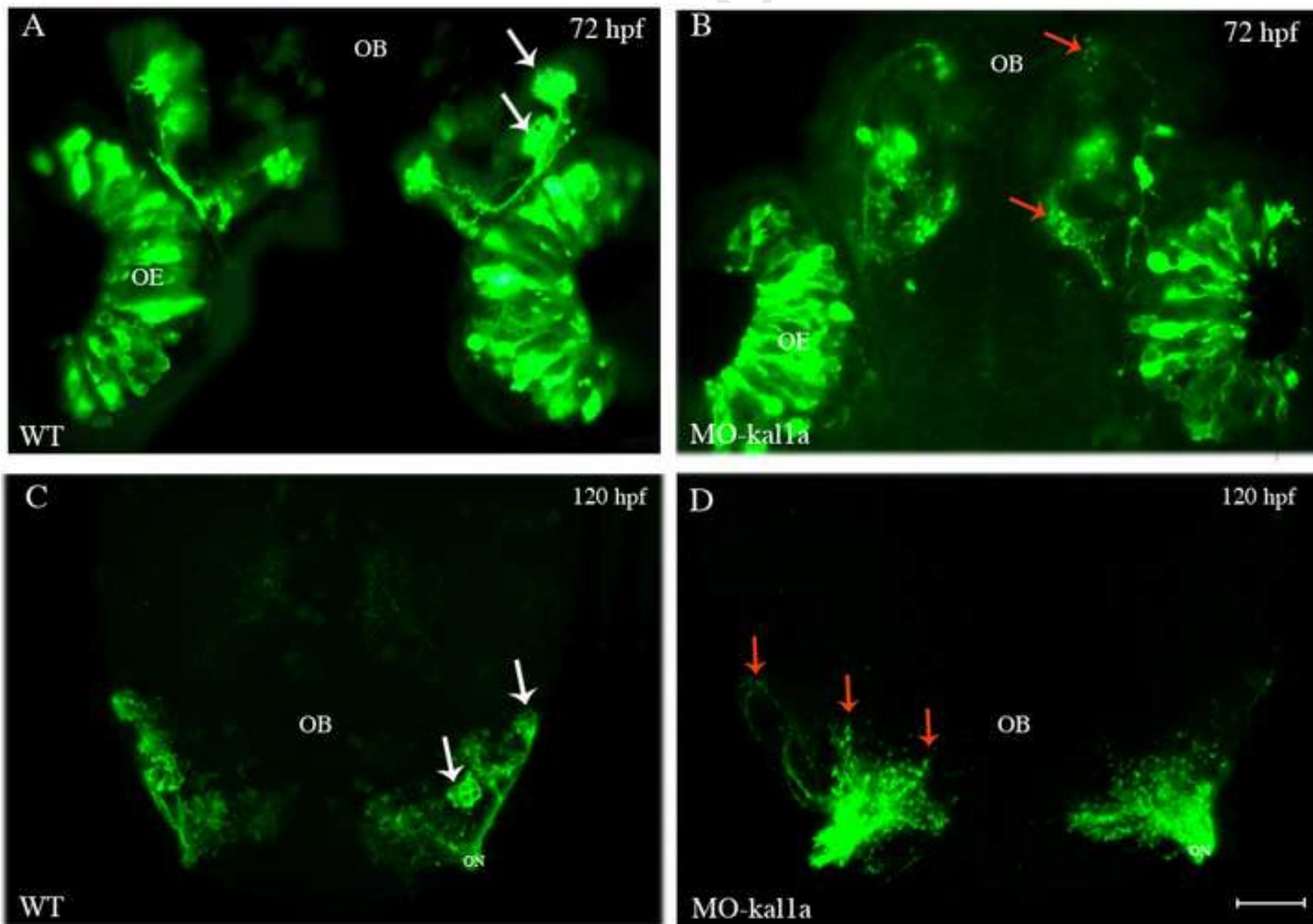
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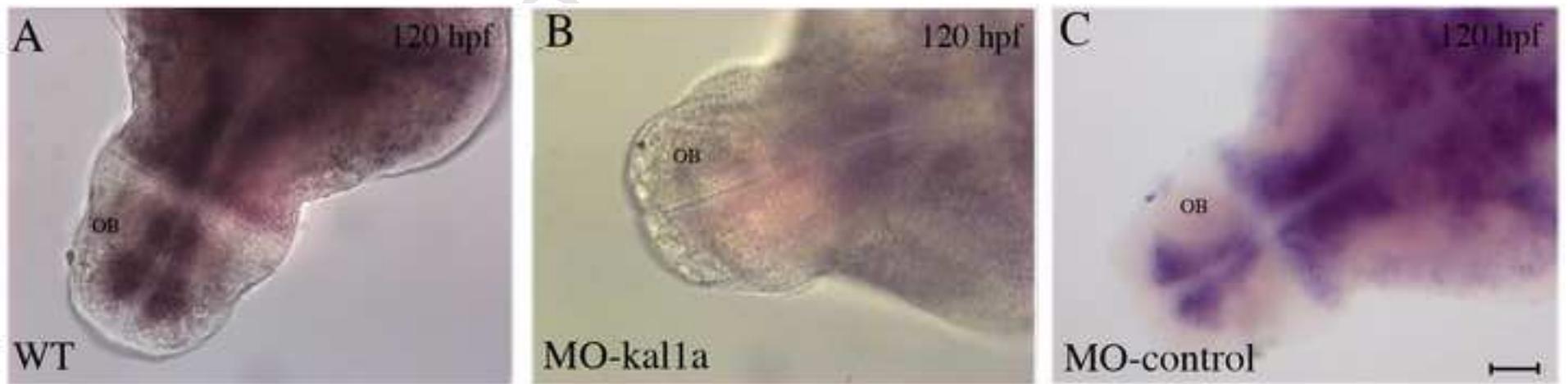
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