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Roles of *Wnt8a* during formation and patterning of the mouse inner ear

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ABSTRACT

Fgf and Wnt signalling have been shown to be required for formation of the otic placode in vertebrates. Whereas several Fgfs including *Fgf3*, *Fgf8* and *Fgf10* have been shown to participate during early placode induction, Wnt signalling is required for specification and maintenance of the otic placode, and dorsal patterning of the otic vesicle. However, the requirement for specific members of the Wnt gene family for otic placode and vesicle formation and their potential interaction with Fgf signalling has been poorly defined. Due to its spatiotemporal expression during placode formation in the hindbrain *Wnt8a* has been postulated as a potential candidate for its specification. Here we have examined the role of *Wnt8a* during formation of the otic placode and vesicle in mouse embryos. *Wnt8a* expression depends on the presence of *Fgf3* indicating a serial regulation between Fgf and Wnt signalling during otic placode induction and specification. *Wnt8a* by itself however is neither essential for placode specification nor redundantly required together with *Fgfs* for otic placode and vesicle formation. Interestingly however, *Wnt8a* and *Fgf3* are redundantly required for expression of *Fgf15* in the hindbrain indicating additional reciprocal interactions between Fgf and Wnt signalling. Further reduction of Wnt signalling by the inactivation of *Wnt1* in a *Wnt8a* mutant background revealed a redundant requirement for both genes during morphogenesis of the dorsal portion of the otic vesicle.

1. Introduction

Formation of vertebrate inner ear is initiated by the induction of the otic placode. This placode emerges from a panplacodal field surrounding the neural plate present shortly after gastrulation. Within this panplacodal field the expression of competence factors is required that will allow a response to inductive signals from surrounding tissues (Ohyama et al., 2006; Schlosser, 2006; Streit, 2007). Having reached this competence the placodal ectoderm is now prepared to receive inducing signals that will specify the identity of particular placodes. In the case of the inner ear placode a first step towards otic fate is taken by the induction of the so-called pre-otic field or otic-epibranchial progenitor domain by Fgfs (Freter et al., 2008; Ladher et al., 2010). Gene inactivation experiments in the mouse have shown that the formation of this domain

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depends on the presence of *Fgf3* together with either *Fgf10* of *Fgf8* (Alvarez et al., 2003; Ladher et al., 2005; Wright and Mansour, 2003; Zelarayan et al., 2007). Next this domain will be partitioned in an otic region and an area that will give rise to epidermis or epibranchial placodes (Freter et al., 2008; Groves and Fekete, 2012; Ladher et al., 2010; Ohyama et al., 2006, 2007). While presence of Wnt signalling will promote otic fate within this domain, Fgf signalling favours the formation of the neighbouring epibranchial placodes (Freter et al., 2008). Whereas the roles of individual Fgf members during induction of the pre-otic field has been clearly defined at least during mammalian inner ear induction, which members of the Wnt gene family are involved in the subsequent steps of placode specification and maintenance has not been clarified. Initially experiments in chick embryos postulated a synergy between *Fgf19* (the orthologue of mouse *Fgf15*), and *Wnt8c* (the orthologue of mouse *Wnt8a*) for initiation of otic development (Ladher et al., 2000). Fgf19 expressed in the mesoderm induced *wnt8c* whose expression is restricted to the area of rhombomere 4 during otic placode formation. Notably Wnt8c by itself also induced *Fgf3* expression which was later on shown to be redundantly required together with *Fgf8* or *Fgf10* for induction of the pre-otic field in mammals (Alvarez et al., 2003; Wright and Mansour, 2003). Requirement of *Wnt8a* during otic placode formation was next directly tested in zebrafish embryos (Phillips et al., 2004). These experiments showed that *Wnt8a* was neither necessary nor sufficient for otic placode formation but was required for timely expression of *Fgf3* and *Fgf8* in the hindbrain during otic induction. Vice versa more recently absence of *Wnt8a* expression has been reported in mouse *Fgf3* mutants (Urness et al., 2010). In the same study Fgf3 was shown to induce *Wnt8a* in ectodermal explants. Finally, mouse mutants with increased Fgf signalling due to the disruption of *Sprouty* genes showed an increased domain of *Wnt8a* expression in the hindbrain and an increased sized otic placode (Mahoney Rogers et al., 2011). Therefore these data indicate a mutual interdependence between the expression of *Fgfs* and *Wnt8a* during inner ear formation in different species. To further explore these interactions and address the role of *Wnt8a* directly in mammals we have analysed *Wnt8a* mouse mutants. These mutants show normal early inner ear development. Likewise reduction of Fgf signalling in the *Wnt8a* mutant knockout does not affect otic placode and vesicle formation. Interestingly however, the expression of *Fgf15* is downregulated in the hindbrain of mouse mutants lacking *Wnt8a* and *Fgf3*. To examine the potential involvement of other Wnt gene family members during otic placode specification we examined *Wnt1* and *Wnt6* which showed an expression pattern consistent with a role during this process. Finally, $Wnt1^{-/-}/Wnt8a^{-/-}$ mutants showed a reduced sized endolymphatic duct and sac demonstrating a redundant requirement of both genes for dorsal patterning of the otic vesicle.

2. Results

2.1. Expression of Wnt8a depends on Fgf3 expression

Crossregulation between the expression of *Wnt8a* and members of the *Fgf* gene family has been observed in a variety of species and has been postulated to underlie otic placode formation (Ladher et al., 2000; Mahoney Rogers et al., 2011; Park and Saint-Jeannet, 2008; Phillips et al., 2004). Similar to the zebrafish (Phillips et al., 2004), in the mouse *Fgf3* and *Wnt8a* are coexpressed in the hindbrain during otic placode induction (Fig. 1). At the onset of somitogenesis coinciding with the formation of the pre-otic field (Jayasena et al., 2008; Ohyama et al., 2006) *Wnt8a* is expressed throughout the embryo with the exception of the anterior headfold region (Fig. 1A). Subsequently during placode formation *Wnt8a* is maintained in an area of the developing hindbrain corresponding to rhombomere 4 (r4) and the primitive streak region (Fig. 1B, D and F). *Fgf3* initially shows expression in the tail region and throughout the rhombencephalon with the highest expression levels present in r4, r5 and r6, and is later maintained in r5 and r6 (Figs. 1C, E and 3I). Interestingly, loss of *Fgf3* expression in *Fgf3* mouse mutants leads to the absence of *Wnt8a* expression throughout the embryo (Fig. 1G and H; Urness et al., 2010). Therefore *Fgf3* expression is required for *Wnt8a* expression in mouse indicating that *Wnt8a* signals may indeed be involved in placode formation in this species.

2.2. Loss of Wnt8a does not affect inner ear placode formation

Inactivation of *Wnt8a* has been reported to result in viable mice but no further characterization of the mutant animals has been described (van Amerongen and Berns, 2006). To examine the requirements of *Wnt8a* for mouse inner ear development its gene was deleted by homologous recombination (see methods). The entire *Wnt8a* coding region was replaced by a neomycin resistance gene and a *lacZ* reporter gene (Fig. 2A). Fidelity of the targeting event was demonstrated by PCR and absence of *Wnt8a* expression in homozygous $Wnt8a^{-/-}$ mutant embryos was confirmed through whole-mount RNA in situ hybridisation (Fig. 2B and C). Embryos carrying the knockout allele showed *lacZ* staining in the hindbrain and the primitive streak region at embryonic day 8 reflecting the endogenous pattern of *Wnt8a* expression (Fig. 2D). However the *lacZ* reporter staining was found to be rather patchy indicating that the *Wnt8a* promoter did not drive *lacZ* expression in all recombined cells.

We next monitored if loss of *Wnt8a* expression may influence formation of the otic placode or vesicle. Expression of *Dlx5*, *Pax2* and *Sox9* all of which have been shown to depend on Wnt signalling during otic placode formation (Ohyama et al., 2006; Saint-Germain et al., 2004) was unaltered in *Wnt8a* mutants (Fig. 3A–F). Likewise expression of *Lunatic fringe* (*Lfng*) a component of the Notch signalling pathway which also has been shown to be regulated by Wnt signalling (Jayasena et al., 2008) was maintained in *Wnt8a* mutant embryos (Fig. 3G and H). Finally, expression of additional neurosensory markers of the developing inner ear such as *NeuroD* (Alsina et al., 2009) and *N-myc* (Dominguez-Frutos et al., 2011) was unaffected in the absence of *Wnt8a* (Supplementary Fig. S1).

Since *Wnt8a* is specifically expressed in the hindbrain in r4 during otic induction we also examined if genes expressed in this area may be affected by its loss. *Hoxb1* which is also expressed in r4 was unaltered in *Wnt8a* mutant embryos (Fig. 3K and L). *Fgf15* which with the exception of r3 is

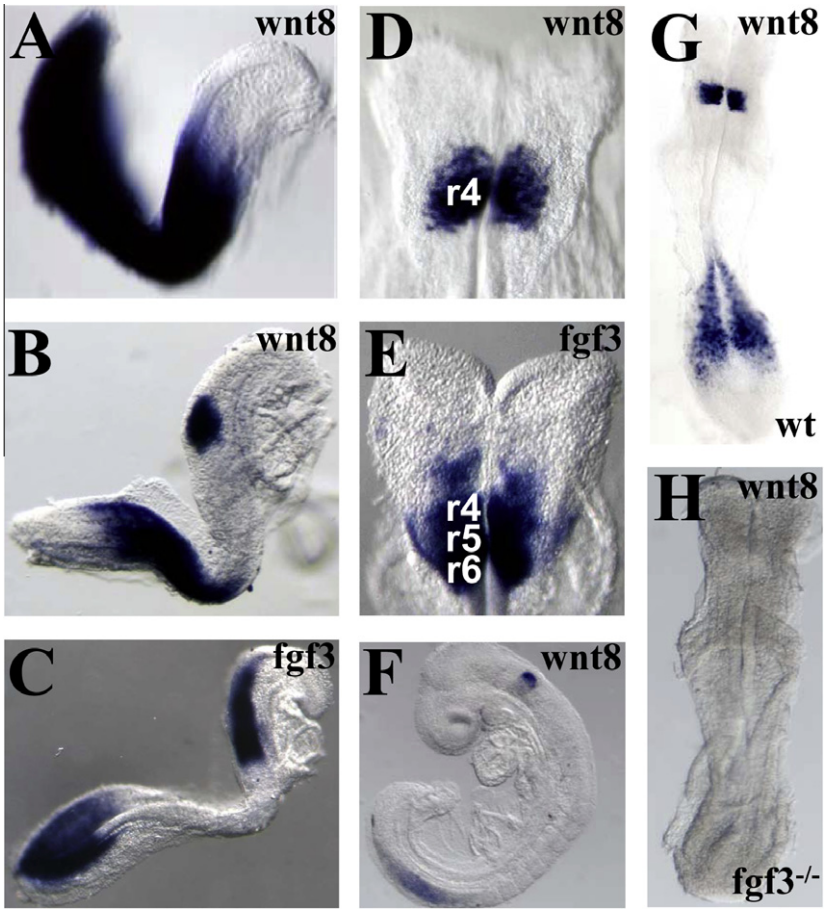

Fig. 1 – Expression of *Wnt8a* and *Fgf3* during inner ear formation revealed by whole mount in situ hybridization. (A, B, D, F–H) Expression of *Wnt8a* at the one somite stage (ss) is detected throughout the embryo with the exception of the headfold region (A). (B) At 3ss *Wnt8a* is observed in the primitive streak and the posterior hindbrain. Within the posterior hindbrain *Wnt8a* is found in the region corresponding to rhombomere 4 (r4) at 5ss (D) and 15ss (F). (G and H) Loss of *Wnt8a* expression in 7ss *Fgf3*$^{-/-}$ mutants (H) is observed throughout the embryo compared to wild-type (wt). (F and E) *Fgf3* is expressed in the tail and throughout the hindbrain at 7ss.

expressed throughout the hindbrain was found to be maintained (Fig. 4A–D). However especially within the more posterior hindbrain *Fgf15* expression was found to be reduced in *Wnt8a* mutants compared to wild-type embryos (Fig. 4A and D). Finally we examined *Fgf3* expression which during otic placode formation initially shows a broad expression throughout the hindbrain before being restricted to rhombomere 5 and 6. A similar pattern of *Fgf3* expression was observed in both control and *Wnt8a* mutants (Fig. 3I and J).

2.3. *Loss of Fgf3 and Wnt8a leads to downregulation of Fgf15*

To identify potential redundant requirements between Fgf and Wnt signalling we created double mutants for *Fgfs* and *Wnt8a*. We first asked if loss of *Fgf3* in a *Wnt8a* mutant background may influence otic induction. Similar to *Wnt8a*$^{-/-}$ mutants, *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ mutants maintained expression of *Pax2*, *Dlx5* and *Lfng* during otic vesicle formation (Fig. 4F, G, J, K, M and N and data not shown). Likewise hindbrain expression of *Hoxb1* in rhombomere 4 was maintained in *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ mutants (Fig. 4H and I). However, examination of hindbrain expression of *Fgf15* now revealed a further downregulation of this gene in the hindbrain of *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ double mutants compared to *Wnt8a*$^{-/-}$ single mutants which resulted in only trace amounts of *Fgf15* expression in r4 (Fig. 4E).

Fgf3 is redundantly required for otic placode induction together with other *Fgf* members such as *Fgf8* (Ladher et al., 2005; Zelarayan et al., 2007). We recently demonstrated that *Fgf8* in the mesoderm is required together with *Fgf3* for inner ear induction (Dominguez-Frutos et al., 2009). We thus next addressed the question if *Wnt8a* expression regulated by *Fgf3* may be an essential component during otic placode formation acting in redundancy with *Fgf8*. Using the *Mesp1*Cre trangenic line we created mutant embryos that lacked *Fgf8* in the mesoderm (Dominguez-Frutos et al., 2009) on a *Wnt8a* homozygous null background. These *Wnt8a*$^{-/-}$/*Fgf8*$^{flox/d2,3}$; *MesP1*$^{Cre/+}$ mutants showed normal formation of the otic vesicle and labelling with the otic markers *Dlx5* and *Pax2* (Fig. 4L and O). Likewise normal *Pax2* and *Sox9* expression could be detected in the otic placode of *Wnt8a*$^{-/-}$/*Fgf8*$^{flox/d2,3}$; *MesP1*$^{Cre/+}$ mutants (data not shown). Therefore *Wnt8a* expression regulated by *Fgf3* is not required for otic placode formation.

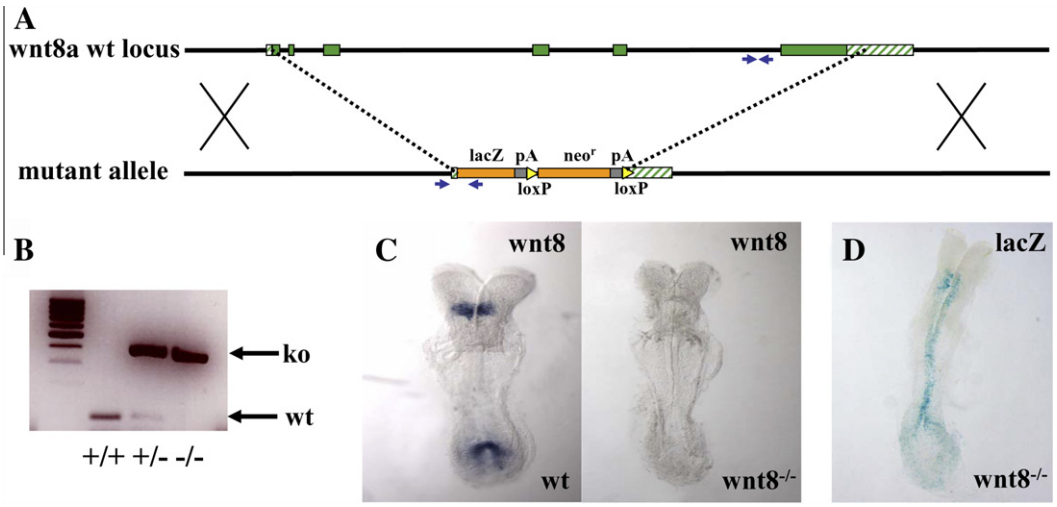

Fig. 2 – Deletion of the *Wnt8a*-coding region in mice. (A) The genomic locus with the exons and coding regions of the *Wnt8a* gene are indicated. The coding region was replaced by a β-galactosidase and a neo^r gene flanked by loxP sites by homologous recombination. (B) PCR analysis using primer pairs indicated by arrows in (A) demonstrating the presence and absence of specific products amplified from the wild-type locus and after generation of the knock out allele. (C) Whole-mount RNA hybridisation analysis of *Wnt8a* expression at embryonic day 8 (E8) in *Wnta8*$^{-/-}$ mutants and a wild-type littermate. (D) *Wnta8*$^{-/-}$ mutant at E8 stained for β-galactosidase activity.

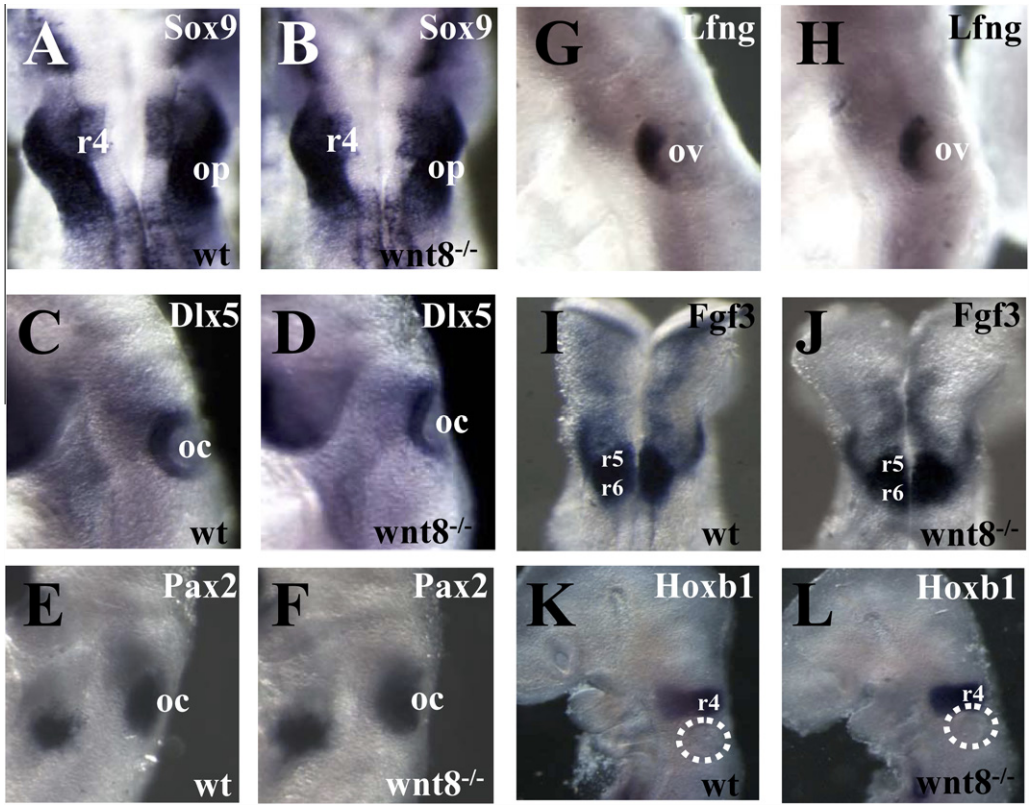

Fig. 3 – Expression of otic and hindbrain markers in *Wnta8*$^{-/-}$ mutants revealed by whole mount RNA in situ hybridisation. Expression of *Sox9* (A and B) in the otic placode (op), *Dlx5* (C and D) and *Pax2* (E and F) in the otic cup (oc), *Lfng* (G and H) in the otic vesicle (ov), and *Fgf3* (I and J) and *Hoxb1* (K and L) in the indicated rhombomeres (r) of the hindbrain.

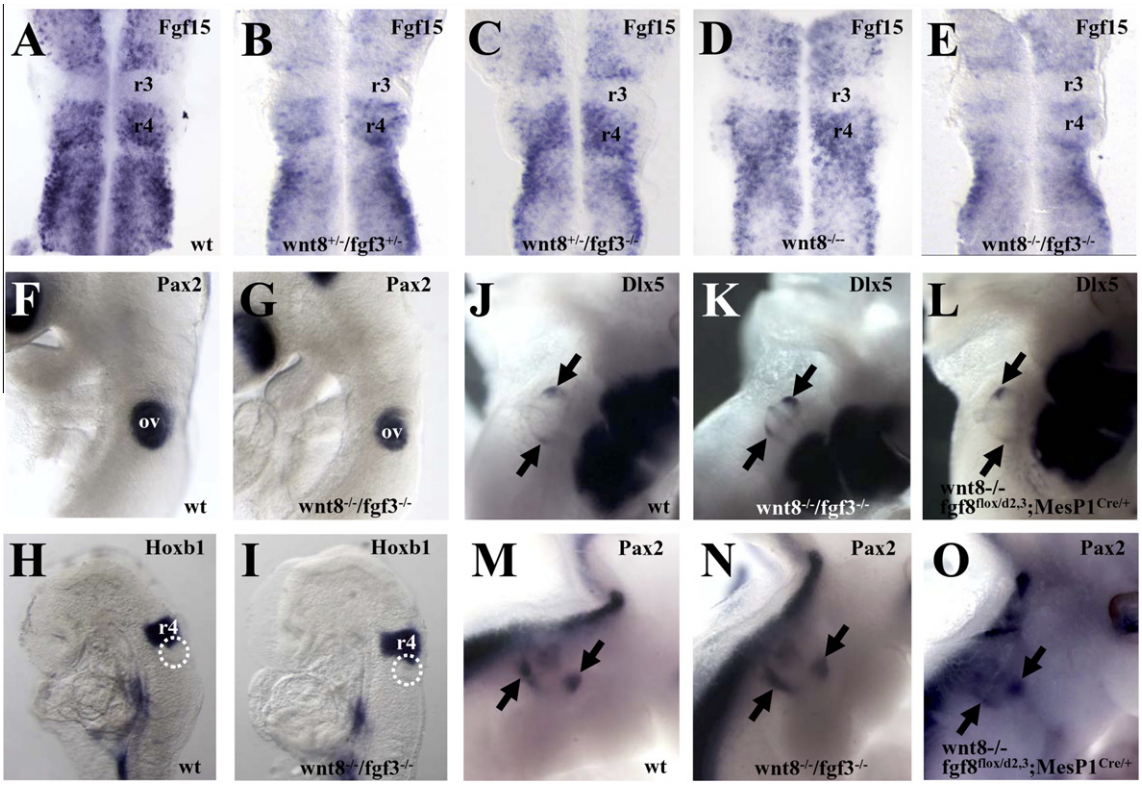

Fig. 4 – Expression of otic and hindbrain markers in *Wnt8a/Fgf* homozygous mutants revealed by whole mount RNA in situ hybridisation. (A–E) Expression of *Fgf15* in flat mounts of the hindbrain of embryos (15ss) with the indicated genotypes. The positions of rhombomere 3 (r3) and rhombomere 4 (r4) are indicated. Note a reduction of *Fgf15* expression in the posterior hindbrain of *Wnt8a*$^{-/-}$ mutants accompanied by a loss of expression in r4 in *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ mutants. Expression of *Pax2* (F and G) in the otic vesicle (ov) and *Hoxb1* (H and I) in r4 in wild-type and *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ mutant embryos. (J–O) Expression of *Dlx5* and *Pax2* in the otic vesicle at E11.5 in wild-type (J and M), *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ (K and N) and *Wnt8a*$^{-/-}$/*Fgf8*$^{flox/d2,3}$; *MesP1*$^{Cre/+}$ mutant embryos (L and O). Arrows indicate the anterior and posterior domains of *Dlx5* expression in (J–L), and the antero-ventral and posterior domains of *Pax2* expression in (M–O).

2.4. *Redundant requirements for Wnt1 and Wnt8a for dorsal patterning of the otic vesicle*

The lack of any phenotypes during inner ear formation in *Wnt8a* mutants indicated that other *Wnt* genes expressed during early inner ear development (Urness et al., 2010) are likely to participate during this process. We first examined expression of *Wnt6* whose expression has been described in the pre-otic field and the dorsal portion of the otic placode of mouse embryos (Jayasena et al., 2008; Urness et al., 2010). *Wnt6* expression was found in the dorsal neuroectoderm and otic placode of wild-type and *Wnt8a*$^{-/-}$ embryos (Fig. 5A–D). We next focussed our interest on *Wnt1* a known regulator of the development of the midbrain-hindbrain boundary (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Next to its expression in the midbrain-hindbrain boundary *Wnt1* expression is also observed in the dorsal portion of the posterior hindbrain during formation of the pre-otic field at the level of rhombomere 4 (Fig. 5E, Li and Joyner, 2001). Upon placode formation and invagination *Wnt1* is broadly expressed throughout the dorsal rhombencephalon and neural tube (Fig. 5F and G). Moreover *Wnt1* has been shown to induce ectopic otic structures upon overexpression in medaka embryos (Bajoghli et al., 2009) and knockdown of *Wnt1* and *Wnt8a* reduces expression of placodal markers in *Xenopus* embryos (Saint-Germain et al., 2004). To examine if *Wnt1*, similary to *Wnt8a*, may also be influenced by *Fgf3* expression in the hindbrain we examined its expression in *Fgf3* null mutants. *Wnt1* expression was found to be maintained in *Fgf3* mutants and was also detected at similar levels in *Wnt8a*$^{-/-}$/*Fgf3*$^{-/-}$ double mutants (Fig. 5H–J).

Similar to the *Wnt8a* mutants described above no defects have been reported during the formation of the inner ear in *Wnt1* mutants (Riccomagno et al., 2005). We therefore next asked if *Wnt1* and *Wnt8a* may act redundantly during inner ear formation and thus created *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ double mutants. Examination of these mutants at embryonic day (E) 11.5 by labelling with *Dlx5* and *Pax2* riboprobes revealed a reduction of the dorsal portion of the endolymphatic duct whereas ventral patterning of the developing otic vesicle appeared normal (Fig. 5K–P). This phenotype was also confirmed at E15.5 when a reduction of the endolymphatic sac which forms at the dorsal portion of the endolymphatic duct during development was observed in *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ double mutants (Fig. 6). Therefore loss of Wnt signalling did not affect inner ear induction but dorsal morphogenesis of the inner ear.

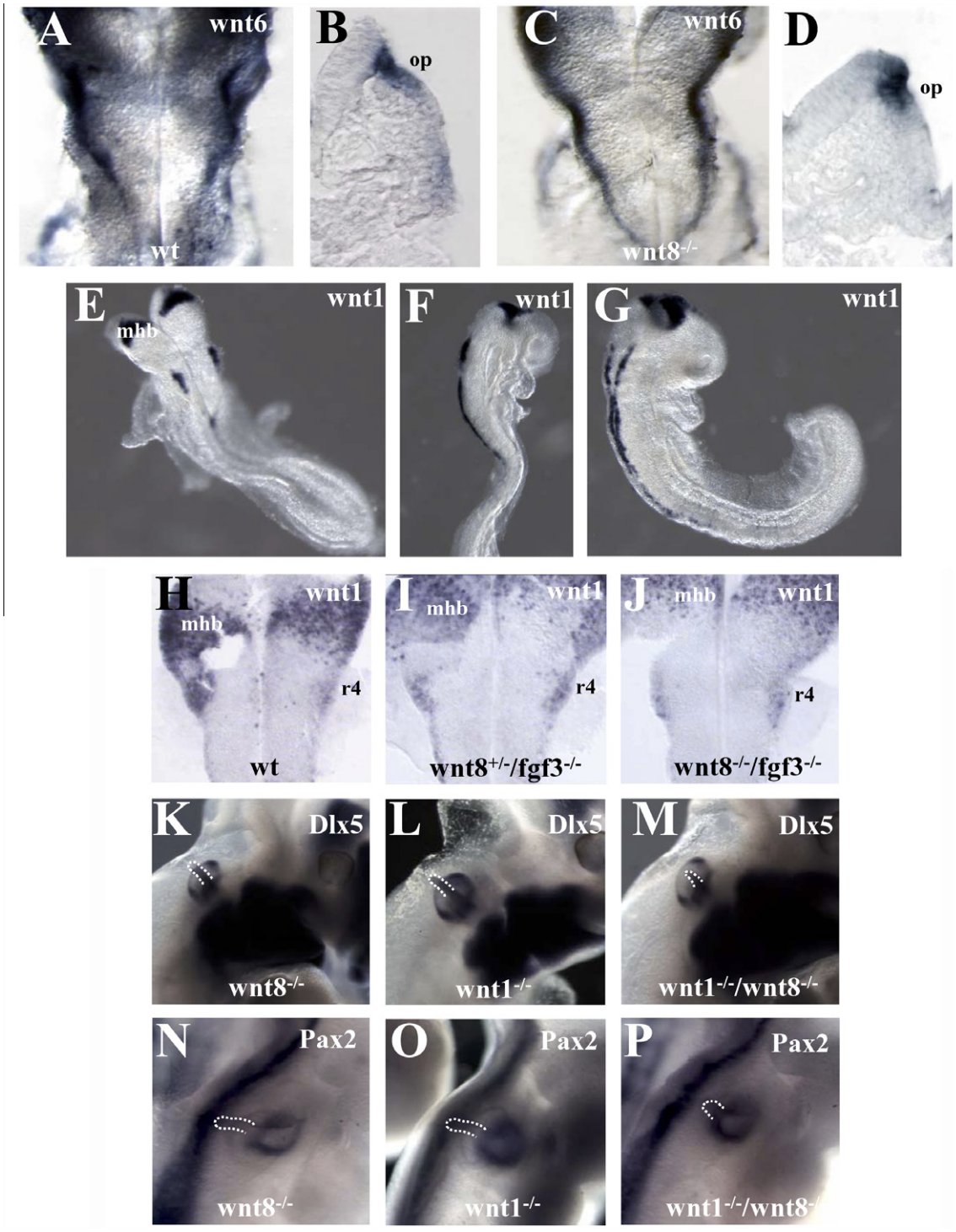

Fig. 5 – Expression of *Wnt1* and *Wnt6* in *Wnt8* mutants and defects in dorsal patterning of the otic vesicle in *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ mutant embryos. (A and C) Dorsal view of *Wnt6* expression at E8.5 and corresponding horizontal sections at the level of the otic placode (B and D) in wild-type and *Wnt8a*$^{-/-}$ embryos. (E–G) Dorsal and lateral views of *Wnt1* expression in embryos at the 5ss (E), 9ss (F) and 13ss (G) reveals expression in the mid-hindbrain boundary (mhb) and the dorsal portion of the neural tube, initially at the level of r4 (E) and later throughout the rhombencephalon and neural tube (F and G). Note also a minor more posteriorly localised domain of *Wnt1* expression in (E). (H–J) Flat mounts of embryos (4ss) with the indicated genotypes labelled with *Wnt1* riboprobes. (K–P) Labelling of embryos at E11.5 with *Dlx5* and *Pax2* riboprobes reveals the presence of a reduced sized endolymphatic duct in *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ mutants. The extension of the endolymphatic duct is indicated by stippled lines. Note that ventral expression of *Pax2* appears unaffected in *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ mutant embryos.

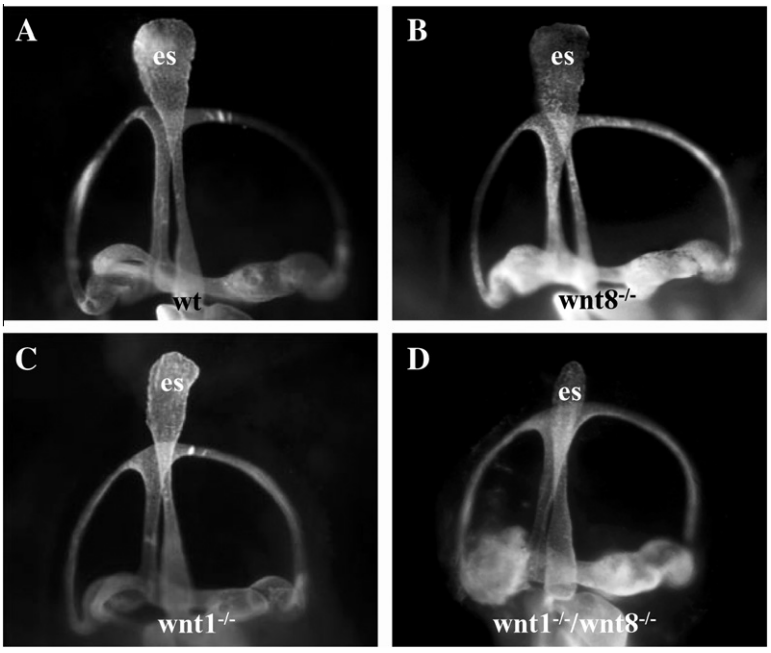

Fig. 6 – Development of the endolymphatic duct and sac in *Wnt* mutants. Paintfilled inner ears of embryos at E15.5 with the indicated genotypes. Note the reduced size of the endolymphatic sac (es) in *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ mutant embryos.

3. Discussion

Sequential signals between members of the Fgf and Wnt gene family have been proposed to act during otic induction and specification in vertebrates (Groves and Fekete, 2012; Ladher et al., 2010; Ohyama et al., 2007). While in the case of *Fgfs*, a direct involvement of *Fgf3*, *Fgf8* and *Fgf10* during otic induction has been clearly defined by loss-of-function experiments in the mouse (Alvarez et al., 2003; Ladher et al., 2005; Wright and Mansour, 2003; Zelarayan et al., 2007), the identity of the Wnt molecules acting during otic specification has not been clarified. Due to its expression pattern in several species and gain-of-function experiments performed in chicken embryos *Wnt8a/Wnt8c* has been proposed as a prime candidate to interact with *Fgfs* during otic placode specification (Groves and Fekete, 2012; Ladher et al., 2010; Ohyama et al., 2007; Urness et al., 2010). Our present results demonstrate that Wnt8a is not essential during this process in mouse.

Wnt8a expression has previously been shown to be delayed during otic placode formation in zebrafish mutants depleted for *Fgf3* and *Fgf8* (Phillips et al., 2004). In the mouse *Wnt8a* expression is strictly dependent on *Fgf3* (present study and Urness et al., 2010) suggesting that Wnt8a might be an essential factor required in redundancy together with other Fgfs such as Fgf10 or Fgf8 to direct otic fate. However, mutants for *Wnt8a* and *Fgf8* show normal formation of the otic placode and vesicle demonstrating that *Fgf3* must control the expression of other genes next to *Wnt8a*.

Although *Wnt8a* is not essential for otic specification, it is required to maintain the expression of *Fgf15*. A similar scenario has been observed in zebrafish where the proper expression of *Fgf3* and *Fgf8* is controlled by *Wnt8a* (Phillips et al., 2004). Unlike *Fgf3* and *Fgf8* however, *Fgf15* is not required for inner ear induction (Wright et al., 2004). Nevertheless the reduced expression of *Fgf15* in *Wnt8a* mutants and absence of *Wnt8a* in *Fgf3* mutants underlines the mutual interdependence between *Fgf* and *Wnt* gene expression during inner ear formation also in mammals.

A lack of a phenotype in *Wnt8a* mutants may be explained by redundancy with other *Wnt* gene family members. We found that *Wnt1* is expressed in the dorsal part of the posterior hindbrain next to which the otic placode is formed in the overlying ectoderm suggesting its possible involvement during early inner ear development. *Wnt1*$^{-/-}$/*Wnt8a*$^{-/-}$ double mutants showed a reduced sized endolymphatic duct and sac demonstrating a redundant requirement of both genes for dorsal patterning of the otic vesicle. Interestingly, a subset of *Fgf3* mutants also show reduction or absence of these structures (Hatch et al., 2007) indicating that the loss of *Wnt8a* in these mutants may underlie this defect. On the other hand *Wnt1*$^{-/-}$/*Wnt3a*$^{-/-}$ double mutants lack expression of the dorsal markers *Dlx5* and *Gbx2* and show a complete disruption of dorsal patterning (Riccomagno et al., 2005). As previously postulated these data confirm that different *Wnt* genes are redundantly required for the specification of the otic placode and patterning and morphogenesis of the dorsal otic vesicle (Groves and Fekete, 2012; Hatch et al., 2007; Ladher et al., 2010; Ohyama et al., 2007). Alternatively species-specific differences may exist which might lead to altered roles for Wnts and Fgfs during placode formation in each species. The present evidence in mouse and chicken embryos suggests that Fgfs act as inducers whereas Wnt signalling is rather involved in placode specification and maintenance (Alvarez et al., 2003; Dominguez-Frutos et al., 2009; Freter et al., 2008; Groves and Fekete, 2012; Ladher et al., 2000, 2005; Ohyama et al., 2006; Wright and Mansour, 2003; Zelarayan et al., 2007). In mouse embryos *Wnt* activity in the otic placode region is only observed after its induction has been initiated and loss of Wnt

signalling leads to defects in specification of the dorsal portion of the otic placode and vesicle (Ohyama et al., 2006; Riccomagno et al., 2005). Likewise, in chicken embryos blockade of Wnt signalling leads to a loss of otic placode markers during otic specification but not during initial induction which depends on Fgfs (Freter et al., 2008). Therefore in these cases Fgf and Wnt signalling appear to act sequentially. In the zebrafish however, although reduced sized otic vesicles form in *Wnt8a* morphants, onset of *Fgf3* and *Fgf8* expression and the preotic marker *Pax8* is delayed (Phillips et al., 2004). These data show that in this species *Wnt8a* at least indirectly participates in otic induction by controlling the timely expression of *Fgfs* required for this process. A cooperation between Wnt and Fgf signals has also been observed in *Xenopus*. In this case different combinations of morpholinos directed against *Fgf3*, *Fgf8*, *Wnt1* or *Wnt8* were shown to reduce expression of the placodal markers *Pax8* and *Sox9* (Saint-Germain et al., 2004). Vice versa misexpression of *Fgf8* in combination with *Wnt1* in animal caps was shown to induce *Pax8* expression. Moreover in medaka embryos *Wnt1* misexpression alone lead to the formation of ectopic otic vesicles (Bajoghli et al., 2009). Therefore in certain species Wnt signalling may also play a more direct role during placode induction. Possibly in lower vertebrates such as medaka, zebrafish or frog where expression of *Wnt* and *Fgf* genes often overlap within the hindbrain during inner ear induction a more direct interaction between both gene families is facilitated. In contrast in birds and mammals *Fgf* genes involved in otic induction such as *Fgf8*, *Fgf10* or *Fgf19* are preferentially expressed in other tissues such as the mesoderm or endoderm. This spatial separation between the expression domains of *Fgf* genes versus *Wnt* genes localised in the hindbrain may also reflect a more functional specialization, *Fgfs* being required for induction whereas *Wnt* genes rather promoting otic specification and patterning.

4. Experimental procedures

4.1. Generation of Wnt8a-deficient mice

Wnt8a-deficient mice were designed and developed by VelociGene technology (Valenzuela et al., 2003). In brief, the *Wnt8a* gene was replaced by a reporter-selection cassette, which consists of a β-galactosidase (*LacZ*) gene and a neomycin resistance gene (see Fig. 2A). The knockout/reporter construct was created by bacterial homologous recombination into a bacterial artificial chromosome encoding *Wnt8a*. The construct deletes 5486 bp between positions 34702008–34707493 of Chromosome 18 that contains the entire coding region of *Wnt8a*. The knockout/reporter construct was electroporated into C57BL/6-derived ES cells. Targeted clones were identified by Taqman screening, using two probes flanking the *Wnt8a* gene as loss-of-allele probes. Chimeric mice were generated by microinjecting targeted ES clones into C57BL/6 embryos. Mice were initially identified as heterozygotes and homozygotes by PCR using probes for the *LacZ* gene and *Wnt8a* (see Fig. 2B). The mutant allele was detected using a primer flanking the deleted region 5′ primer, GGTAGGAGACCTGCTTCAGC and the *LacZ* 3′ primer GTCTGTCCTAGCTTCCTCACTG producing a band of 361 bp. The wild-type allele was detected using primers 5′GCTTCCGTCATCTTCTTAGCAC and 3′ GGGCACTCCTAA CCCTGTC amplifying a band of 99 bp from the *Wnt8a* gene. The absence of *Wnt8a* in homozygous mutant mice was confirmed by RNA in situ hybridisation (see Fig. 2C). *Wnt8a*$^{-/-}$ mutants were viable and fertile and showed no apparent abnormalities. For routine genotyping the following primers were used: s-TCACCAGCACAACTACGCCG and as-GTCTG TCCTAGCTTCCTCACTG and as-CAGCATTAACAAGTGT CCCATGG which resulted in the generation of 265 bp (wt) and 361 bp (mutant) products. Cycling was conducted as follows: 99 °C and 94 °C for 5 min each followed by 30 cycles of the following: 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 30 s.

4.2. RNA in situ hybridization

RNA whole-mount in situ hybridisation, β-galactosidase staining and the sectioning of stained embryos has been described previously (Alvarez et al., 2003). The riboprobes corresponding to *Wnt8a* (Bouillet et al., 1996), *Wnt6* (Urness et al., 2010), *Wnt1* (Wilkinson et al., 1987), *Fgf15* (Wright et al., 2004) and *Hoxb1* (Wilkinson et al., 1989) have been described. All other riboprobes used in this study have been referred to previously (Alvarez et al., 2003; Zelarayan et al., 2007).

4.3. Paint-filling of inner ears

Paint-filling was performed following the protocol described by Kiernan (Kiernan, 2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mod.2012.09.009.

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