

# Dynamic temporal requirement of *Wnt1* in midbrain dopamine neuron development

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## SUMMARY

*Wnt1*-expressing progenitors generate midbrain dopamine (MbDA) and cerebellum (Cb) neurons in distinct temporal windows and from spatially discrete progenitor domains. It has been shown that *Wnt1* and *Lmx1a* participate in a cross-regulatory loop that is utilized during MbDA neuron development. However, *Wnt1* expression dynamically changes over time and precedes that of *Lmx1a*. The spatial and temporal requirements of *Wnt1* in development and specifically its requirement for MbDA neurons remain to be determined. To address these issues, we generated a conditional *Wnt1* allele and temporally deleted *Wnt1* coupled with genetic lineage analysis. Using this approach, we show that patterning of the midbrain (Mb) and Cb by *Wnt1* occurs between the one-somite and the six- to eight-somite stages and is solely dependent on *Wnt1* function in the Mb, but not in the Cb. Interestingly, an *En1*-derived domain persists after the early deletion of *Wnt1* and mutant cells express OTX2. However, the *En1*-derived *Wnt1*-mutant domain does not contain LMX1a-expressing progenitors, and MbDA neurons are depleted. Thus, we demonstrate an early requirement of *Wnt1* for all MbDA neurons. Subsequently, we deleted *Wnt1* in the ventral Mb and show a continued late requirement for *Wnt1* in MbDA neuron development, but not in LMX1a-expressing progenitors. Specifically, *Wnt1* deletion disrupts the birthdating of MbDA neurons and causes a depletion of MbDA neurons positioned medially and a concomitant expansion of MbDA neurons positioned laterally during embryogenesis. Collectively, our analyses resolve the spatial and temporal function of *Wnt1* in Mb and Cb patterning and in MbDA neuron development *in vivo*.

**KEY WORDS:** *Wnt1*, Conditional knockout, Dopamine neurons, Genetic inducible fate mapping, Midbrain, Neural development, Mouse

## INTRODUCTION

The midbrain (Mb) comprises the superior colliculus, which integrates attention and visual stimuli, and the inferior colliculus, which is an obligate auditory processing center. These dorsal Mb (d.Mb) structures are adjacent to the cerebellum (Cb), which controls motor behaviors. The ventral Mb (v.Mb) contains molecularly, physiologically and biochemically distinct neurons that control complex functions. Of particular interest are the Mb dopamine (MbDA) neurons because they modulate brain function and are centrally involved in schizophrenia, addiction and Parkinson's disease. During embryogenesis, MbDA neurons are derived from multiple lineages originating within the mesencephalon (mes), which is the Mb primordium (Zervas et al., 2004; Joksimovic et al., 2009; Brown et al., 2011; Hayes et al., 2011; Blaess et al., 2011). The molecular identity of the mes is based on a combinatorial code of transcription factors and is patterned by signaling molecules, including WNT1 (reviewed by Zervas et al., 2005). We investigated *Wnt1*, which is initially expressed broadly in the mes, but becomes restricted to distinct domains over time (Wilkinson et al., 1987; Echelard et al., 1994; Zervas et al., 2004). Mice with a null allele of *Wnt1* or with a spontaneous mutation in *Wnt1* do not form the Mb and Cb, demonstrating the importance of WNT1 for these domains (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas et al., 1991; Bally-Cuif et al., 1995; Ellisor et al., 2012). WNT1 functions through  $\beta$ -catenin-mediated WNT

signaling and  $\beta$ -catenin regulates cell cycle exit and MbDA neuron development (Tang et al., 2009; Tang et al., 2010). In addition,  $\beta$ -catenin binds to the promoter of *Lmx1a*, which is proposed to be a determinant of MbDA neurons (Andersson et al., 2006; Chung et al., 2009). However, all WNTs that use canonical signaling converge on  $\beta$ -catenin, which precludes an understanding of the specificity of WNT ligand involvement in MbDA neuron development *in vivo*. Genetic inducible fate mapping (GIFM) shows that distinct spatial and temporal epochs underpin *Wnt1* lineage contribution to MbDA neurons and to Cb neurons (Brown et al., 2011; Hagan and Zervas, 2012). Furthermore, dynamic oscillatory genetic networks in human neuronal progenitors are influenced by WNT1 (Wexler et al., 2011). However, the functional requirement of WNT1 in developmental processes is unresolved. Thus, we generated a conditional knockout allele of *Wnt1* that we coupled with GIFM to uncover distinct spatial and temporal requirements for *Wnt1* in patterning the Mb and Cb, controlling ventral mesencephalon (v.Mes) progenitors and regulating cell cycle exit during MbDA neuron development.

## MATERIALS AND METHODS

### *Wnt1* conditional knockout targeting construct (*pWnt1<sup>neoflox</sup>*)

*Wnt1* conditional knockout constructs were generated by recombineering (supplementary material Fig. S1) (Liu et al., 2003). Briefly, we captured *Wnt1* and modified it by placing a *loxP*-PGK-*gb2-neo-loxP* cassette between the third and fourth exons (*LoxP*) (supplementary material Fig. S1A,B, Table S1). Recombinants were determined by positive selection followed by pKS-Cre removal of the *Neo* cassette. A second *FRT*-PGK-*gb2-neo-FRT-loxP* cassette was inserted between the first and second exons (*LoxP-FRT*; Table 1) and was used for selection in embryonic stem (ES) cells. The targeting vector was designated as *pWnt1<sup>neoflox</sup>* (supplementary material Fig. S1B). Plasmids were verified by PCR, restriction digestion and sequencing. The recombination potential of *pWnt1<sup>neoflox</sup>* was determined by expressing it in *Escherichia coli* cells electroporated with pKS-Cre, resulting in the deletion of exons 2 and 3 (supplementary material Fig. S1C).

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### Gene targeting and conditional gene manipulations

JM8.F6 ES cells of C57Bl/6 origin (generously provided by Dr Bill Skarnes) (Pettitt et al., 2009) were electroporated with 20 µg of linearized *pWnt1<sup>neoflox</sup>*. G418-resistant clones were confirmed by PCR screening for the *Neo* cassette (primers 3, 5) and positive clones were screened for the absence of the *AMP* cassette by PCR (primers 9, 10). Clones were also screened for the downstream *loxP* site (primers 1, 2). Primer sequences are provided in supplementary material Table S2. Two correctly targeted ES clones, PL1G10 and PL1G12, were karyotyped as 95% normal and used for blastocyst injection. Correctly targeted *Wnt1<sup>neoflox/+</sup>* founders were derived from germ line chimeras and bred to *Flpe* mice (The Jackson Laboratory #003946) to delete the *Neo* cassette. Subsequent matings eliminated the *Flpe* allele to generate the *Wnt1<sup>lox</sup>* allele (*Wnt1<sup>fl</sup>*). *Wnt1<sup>fl/+</sup>* or *Wnt1<sup>fl/fl</sup>* mice were bred with the following lines: (1) *En1<sup>Cre</sup>* for early [starting at embryonic day (E) 8.0] cumulative recombination (Kimmel et al., 2000; Chi et al., 2003; Ellisor et al., 2009); (2) *Gbx2<sup>CreER-ires-eGFP</sup>* (Chen et al., 2009; Luu et al., 2011) and (3) *Wnt1-CreER* plus tamoxifen for temporal control of recombination (Zervas et al., 2004; Brown et al., 2011); (4) *Shh<sup>Cre</sup>* for cumulative recombination starting at E9.0-9.5 (Harfe et al., 2004; Hayes et al., 2011). Tamoxifen takes 6 hours to initiate recombination and lasts for 24-30 hours (reviewed by Ellisor et al., 2009; Brown et al., 2009). Genotyping was carried out as previously described (Ellisor et al., 2009). *Rosa26<sup>lox-STOP-lox-tdTomato</sup>* (*R26<sup>tdTomato</sup>*) mice were used for lineage tracing and *Wnt1-Venus* mice were used to detect *Wnt1* expression (Madisen et al., 2010; Brown et al., 2011; Ellisor et al., 2012). All *Cre* and reporter lines in this study have been validated and described elsewhere (Kimmel et al., 2000; Li et al., 2002; Zervas et al., 2004; Madisen et al., 2010; Brown et al., 2011; Hagan and Zervas, 2012; Hayes et al., 2011). Mice were housed and handled in accordance with Brown University Institutional Animal Care and Use Committee guidelines.

### In situ hybridization, β-galactosidase (β-gal) histochemistry and immunofluorescence immunocytochemistry

Full details of these experimental protocols have been previously published (Ellisor et al., 2009; Brown et al., 2011; Hagan and Zervas, 2012). Primary antibodies for marker analysis were: anti-tyrosine hydroxylase (TH; Chemicon, 1:500), anti-5-hydroxytryptamine (5-HT; Jackson ImmunoResearch, 1:500), anti-OTX2 (Abcam, 1:250), anti-NURR1 (Santa Cruz, 1:200), anti-LMX1a (Michael German, UCSF, 1:1000), anti-GFP (Nacalai Tesque, 1:500) and anti-dsred (1:500, Clontech). Secondary antibodies (Molecular Probes, 1:500) were: donkey anti-rabbit Alexa 555, donkey anti-rat Alexa 488, donkey anti-rabbit IgG-Alexa488, donkey anti-goat IgG-Alexa488, donkey anti-rabbit IgG-Alexa 350.

### Cell cycle analysis

Pregnant dams were given 20 mg/kg 5-ethynyl-2'-deoxyuridine (EdU) by intraperitoneal injection (Wang et al., 2011) and sections were labeled for Ki67 (1:100; rat monoclonal IgG2a; Dako, cat #M7249), TH (1:500; rabbit polyclonal IgG; Millipore-Chemicon, cat #AB152), RFP (1:1000; chicken polyclonal IgG; VWR, cat #RL600-901-379), EdU (Click-iT EdU Alexa Fluor 647 Imaging Kit; Invitrogen, cat #C10340) and Hoechst. EdU staining was carried out following the manufacturer's instructions. Antigen retrieval was carried out for Ki67 immunolabeling by boiling sections in R-Buffer A (Electron Microscopy Sciences) and neutralizing with 0.1 M borate buffer. Secondary antibodies (1:500) were: Alexa 488 (donkey anti-rat; Invitrogen, cat #A-21208), Alexa 594 (donkey anti-rabbit; Invitrogen, cat #A-11012), Alexa 555 (goat anti-chicken; Invitrogen, cat #A-21437). Sections were imaged using a Zeiss LSM 710 Confocal Laser Scanning Microscope (lasers for excitation at 405 nm, 488 nm, 561 nm, 594 nm and 633 nm) with a 40× water immersion objective (NA 1.20) and a 1.3× zoom. z-series stacks were captured at 1-µm intervals and processed with ZEN 2009 software. Images were acquired with a 34-channel QUASAR detector set to collect emitted light of specific wavelengths. Beam splitters were optimized to collect fluorescent signals: dsred (Alexa 555, imaged using the 561 laser, a collection window of 569-598 nm, and one beam splitter for 488 nm/561 nm); Hoechst and TH [Alexa 594, imaged simultaneously with 594 nm and 405 nm lasers, collection windows at 426-589 nm (Hoechst) and 598-646 nm (Alexa 594), and two beam splitters for 488 nm/594 nm and 405 nm].

Ki67 and EdU [Alexa 488 and Alexa 647 were imaged simultaneously with 488 nm and 633 nm lasers, respectively, collection windows at 501-550 nm (Alexa 488) and 646-695 nm (Alexa 647), and one beam splitter for 488 nm/561 nm/635 nm signal]. Fluorescent signals did not bleed through into other channels. Pinhole settings for image acquisition were 30 µm (405 nm and 594 nm lasers), 34 µm (561 nm laser) and 31 µm (488 nm and 633 nm lasers). The ventricular zone (outer layer of v.Mes) and the differentiated zone (inner layer of v.Mes) at rostral, intermediate and caudal locations were sampled by collecting six z-series stacks: (1) rostral-inner, (2) rostral-outer, (3) intermediate-inner, (4) intermediate-outer, (5) caudal-inner, (6) caudal-outer using a 166 × 166 µm optical dissector (Fig. 6; supplementary material Fig. S9). Three medial, three off-midline and three lateral sagittal sections were identified as described previously (Brown et al., 2011) and sampled to obtain medial-to-lateral slabs. Marker positive cells were counted from *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/+</sup>;R26<sup>tdTomato</sup>* (*n*=5), *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>tdTomato</sup>* or *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>* embryos (*n*=4). Cell counts from animals of matched genotypes were summed (total count) and the total counts for each animal were averaged. A two-tailed *t*-test was used to analyze cell counts and to determine statistical significance (*P*-values are indicated in text).

### Microdissection and fluorescence-activated cell sorting (FACS)

The v.Mes of *Wnt1-Venus* embryos was microdissected as previously described (Brown et al., 2009; Ellisor et al., 2012). Briefly, we isolated the v.Mes under a stereofluorescence microscope. The small domain of YFP-expressing cells was carefully dissected away from the posterior mes 'ring' and dorsal tissue. In addition, d.Mes or whole head was isolated. The isolated tissues were trypsinized using TrypLE Express (Gibco) and DNase (Roche) at 1:1000 for 8 minutes at 37°C. Subsequently, 10% FBS in PBS was added to stop the reaction. Samples were then mechanically dissociated using a 20-gauge needle and 1cc syringe to generate a single cell suspension. Cells were stored on ice until sorting with a FACSaria flow cytometer (BD Biosciences) and analyzed using Diva software (BD Biosciences) at Brown University's Flow Cytometry and Sorting Facility. Population gates were established by setting threshold values based on wild-type littermate control samples and negative control samples obtained from the prosencephalon. Cells were sorted into clean eppitubes containing a small volume of 10% FBS in PBS and spun at 3000 rpm (800 g) for 10 minutes at 4°C. PBS was removed and 100 µl of Trizol (Invitrogen) was added and samples were stored at -80°C until RNA extraction.

### Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

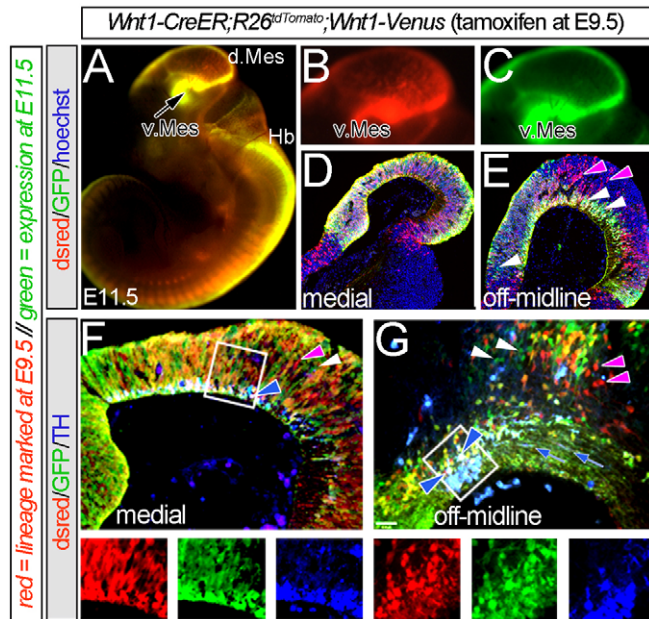
Cells in Trizol were thawed on ice and allowed to sit at room temperature for 5 minutes. Chloroform (25 µl) was added to each tube and shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 4°C for 15 minutes. RNA was transferred to a new tube and isopropanol (50 µl) was added and incubated for 10 minutes at room temperature. Samples were spun at 13,200 rpm (16,100 g) for 15 minutes at 4°C. The RNA pellet was washed with 250 µl of 75% ethanol, spun at 7500 rpm (5100 g) for 5 minutes at 4°C, and resuspended in 20 µl of dH<sub>2</sub>O. RNA was quantified with a nanodrop and assayed for purity with an Agilent Bioanalyzer. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad #170-8890) according to manufacturer's instructions. We used 0.5 µg of RNA template, 4 µl of 5× iScript reaction mix, 1 µl of iScript reverse transcriptase (RT) in a 15 µl reaction. The thermocycle program was 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes. qRT-PCR was performed with SYBR Green (Applied Biosystems #4367659) in MicroAmp 96-well reaction plates. For each primer set, we used a reaction mix of nuclease-free water (7.5 µl), Primer 1 (2.0 µl), Primer 2 (2.0 µl), SYBR Green (12.5 µl). Note that all primers were tested to ensure that they yielded a single amplicon of appropriate size (typically ~200 nucleotides). All samples were processed in triplicate and dissociation curves were compared with those obtained for 18S. Relative RNA levels (average±s.d.) were plotted using Numbers; statistical significance was determined using Student's *t*-test (*P*<0.05). Primer sequences are provided in supplementary material Table S3.

## RESULTS

### Dynamic expression of *Wnt1* in MbDA neuron progenitors

We examined *Wnt1* at well-defined stages of MbDA neuron development (Ang, 2006) and focused on v.Mes progenitors because they are a substantial source of MbDA neurons *in vivo* (Brown et al., 2011). We used a genetics-based approach that has been instructive in assessing dynamic gene regulation in spinal cord (Luu et al., 2011). Specifically, we administered tamoxifen at E9.5 to pregnant females harboring *Wnt1-CreER;R26<sup>tdTomato</sup>;Wnt1-Venus* embryos followed by analysis 48 hours later (Fig. 1). In this short-term GFM experiment, the *Wnt1-CreER;R26<sup>tdTomato</sup>* allelic combination plus tamoxifen marked the *Wnt1*-expressing progenitor pool at E9.5 whereas the *Wnt1-Venus* transgene showed 'current' *Wnt1* expression at the stage of analysis (E11.5). *Wnt1-CreER;R26<sup>tdTomato</sup>;Wnt1-Venus* embryos had *Wnt1* lineage-derived cells (red fluorescence<sup>+</sup>) that were GFP<sup>-</sup> in the dorsal mesencephalon (d.Mes), indicating that *Wnt1* expression in these progenitors had ceased over the 2 days following marking (Fig. 1A-C). By contrast, the *Wnt1* lineage in the v.Mes appeared to be double positive (red fluorescence<sup>+</sup>/GFP<sup>+</sup>), suggesting that *Wnt1* expression in v.Mes progenitors persisted (Fig. 1A-C). We analyzed the v.Mes by double immunolabeling with an anti-dsred antibody to identify the *Wnt1* lineage (dsred<sup>+</sup>, red) and an anti-GFP antibody to identify *Wnt1*-expressing progenitors (GFP<sup>+</sup>, green)

(Fig. 1D,E). In medial sections, much of the *Wnt1* lineage marked at E9.5 continued to express *Wnt1* (dsred<sup>+</sup>/GFP<sup>+</sup>, yellow cells) (Fig. 1D). However, off-midline sections showed heterogeneity of progenitors: *Wnt1*-lineage derived cells closest to the v.Mes flexure were dsred<sup>+</sup>/GFP<sup>+</sup> (Fig. 1E, yellow cells) interspersed amongst cells expressing GFP only (Fig. 1E, white arrowheads). Cells proximal to the ventricle were dsred<sup>+</sup> (Fig. 1E, purple arrowheads). Interestingly, MbDA neurons (tyrosine hydroxylase, TH<sup>+</sup>) at E11.5 were located in the differentiated zone at the v.Mes flexure and were derived from the *Wnt1* lineage and continued to express *Wnt1* (Fig. 1F,G; TH<sup>+</sup>/dsred<sup>+</sup>/GFP<sup>+</sup>, blue arrowheads). In addition, we observed clonal-like cohorts of cells that had expressed *Wnt1* at E9.5 but had ceased to express *Wnt1* by E11.5 (Fig. 1F,G; dsred<sup>+</sup>/GFP<sup>-</sup>, purple arrowheads), and clones that appeared to newly express *Wnt1* (Fig. 1F,G; dsred<sup>-</sup>/GFP<sup>+</sup>, white arrowheads). One caveat of this last group is that some cells may have escaped marking owing to the mosaic nature of CreER-tamoxifen. We observed a similar dynamic regulation when we administered tamoxifen at E8.5 and analyzed E12.5 embryos, with the exception of the marked *Wnt1* lineage being more broadly distributed throughout the mes (data not shown). Thus, there was a cohort of the *Wnt1* lineage that expressed *Wnt1* early and then extinguished *Wnt1* over the following 2-4 days and cells that expressed *Wnt1* early and continued to express *Wnt1*. These findings showed dynamic regulation of *Wnt1* and suggested distinct temporal roles for *Wnt1* in development.



**Fig. 1. Dynamic regulation of *Wnt1* expression.** (A-C) *Wnt1-CreER;R26<sup>tdTomato</sup>;Wnt1-Venus* embryo at E11.5 with *Wnt1* lineage marked at E9.5 (dsred<sup>+</sup>) and 'current' *Wnt1*-expressing progenitors (YFP<sup>+</sup>, green). (D,E) Immunolabeling of the *Wnt1* lineage (dsred<sup>+</sup>, red) and *Wnt1*-expressing progenitors (GFP<sup>+</sup>, green) in the v.Mes. Purple arrowheads indicate progenitors that expressed *Wnt1* at E9.5 and ceased to express *Wnt1* (red cells); white arrowheads indicate progenitors that newly turned on *Wnt1* (GFP<sup>+</sup>); yellow cells are progenitors that expressed *Wnt1* early and continued to express *Wnt1*. (F,G) MbDA neurons (TH<sup>+</sup>, blue) derived from the *Wnt1* lineage (red) continued to express *Wnt1* (green) (TH<sup>+</sup>/dsred<sup>+</sup>/GFP<sup>+</sup>, blue arrowheads). Lower panels in F,G show single channels of MbDA neurons from regions of interest (boxes). Purple arrowheads indicate cells that expressed *Wnt1* early and then ceased to express *Wnt1*; white arrowheads indicate progenitors that newly turned on *Wnt1*.

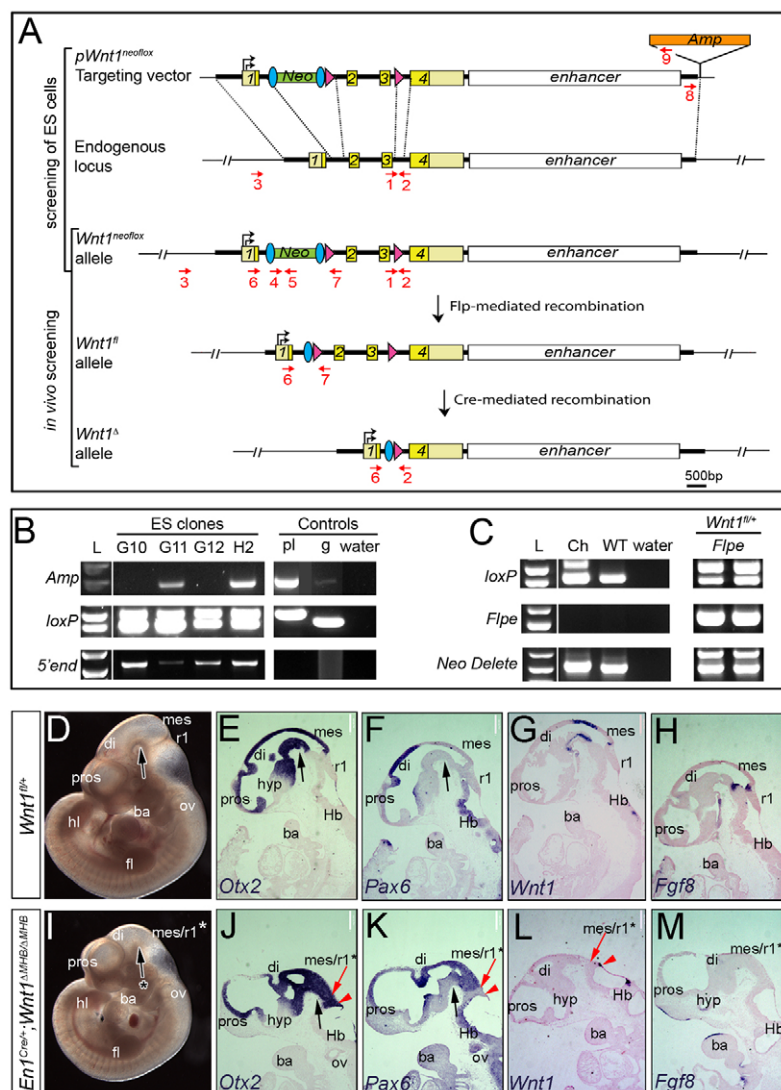
### Generation of a conditional knockout allele of *Wnt1*

We generated a conditional knockout allele of *Wnt1* using recombineering technology (Liu et al., 2003) to address the temporal requirement of *Wnt1* in development (supplementary material Fig. S1). The entire *Wnt1* gene was captured from a bacterial artificial chromosome (BAC) library (supplementary material Fig. S1A) and modified by cloning a loxP site between the third and fourth exons and cloning a FRT-PGK-*gb2-neo-FRT-loxP* cassette between the first and second exons (Fig. 2A; supplementary material Fig. S1B). Linearized *pWnt1<sup>neoflox</sup>* was electroporated into mouse ES cells and clones that had undergone homologous recombination were used to generate chimeras (Fig. 2A,B). *Wnt1<sup>neoflox/+</sup>* founders were bred to *Flpe* mice to remove the *Neo* cassette (Fig. 2A,C). To validate the ability to convert the *Wnt1<sup>fl</sup>* to the deleted allele *in vivo* (designated *Wnt1<sup>Δ</sup>*), we bred *Wnt1<sup>fl/+</sup>* males with *En1<sup>Cre</sup>* females and analyzed the rostral and caudal halves of embryos at the 6- to 8-somite stage (*n*=3) by PCR, which confirmed the *Wnt1<sup>Δ</sup>* allele in embryos that inherited *En1<sup>Cre</sup>* (supplementary material Fig. S2).

### Conditional *Wnt1* deletion disrupts mesencephalon and rhombomere 1 patterning

We generated and bred *En1<sup>Cre</sup>;Wnt1<sup>fl/+</sup>* to *Wnt1<sup>fl/+</sup>* mice to conditionally delete *Wnt1* early in midbrain-hindbrain (MHB) development, consistent with *En1* expression and *En1<sup>Cre</sup>*-mediated recombination (Li et al., 2002; Chi et al., 2003; Ellisor et al., 2009). Operationally and for clarity of discussing the multiple *Cre* lines used in this study, we designated the deleted allele in the MHB as *Wnt1<sup>ΔMHB</sup>*. *Wnt1<sup>fl/+</sup>* and *Wnt1<sup>+/+</sup>* embryos were morphologically normal at E10.5 (*n*=4) (Fig. 2D; supplementary material Fig. S3). By contrast, *En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/ΔMHB</sup>* conditional mutant littermates (*n*=3) had a severe deletion of the putative mes and rhombomere 1 (r1) (Fig. 2I; supplementary material Fig. S3). We assayed sagittal sections from controls and conditional mutants by molecular





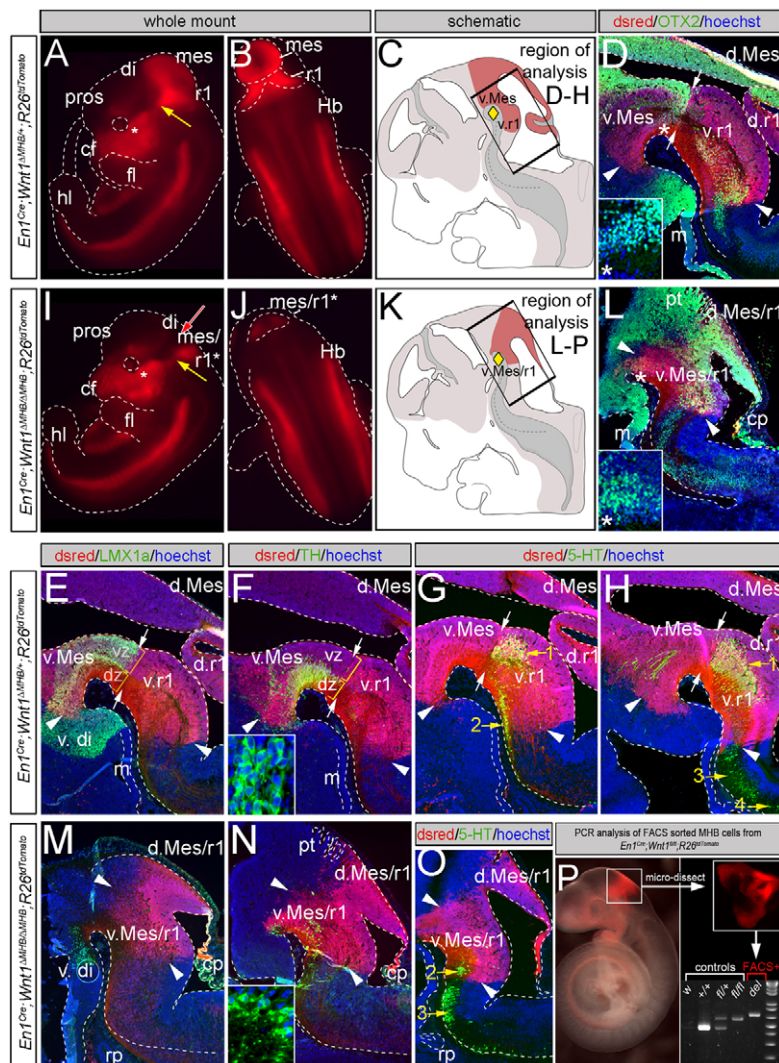
**Fig. 2. *Wnt1* conditional knockout allele and conditional deletion of *Wnt1* in the Mb and Hb.** (A) The targeting vector contained *Frts* (blue ovals) and *loxP* (pink triangles) sites and an *Amp* cassette (orange rectangle). Homologous recombination replaced endogenous *Wnt1* and *Flpe* removed the *Neo* selection cassette (green rectangle). Cre-mediated recombination generates a *Wnt1*<sup>Δ</sup> allele. (B) PCR screening of ES cell clones: G10 and G12 did not contain the *Amp* cassette (amplified by primers 9,10), were heterozygous for the downstream *loxP* site (primers 1,2) and were positive for *Neo* (primers 3,5); G11 and H2 did not have the correct profile and were not used. Control plasmid (pl, pWnt1<sup>neoflox</sup>) had an *Amp* cassette and downstream *loxP* site. Genomic DNA (g) produced an amplicon corresponding to *Wnt1* (primers 1,2). (C) *Wnt1*<sup>fl/+</sup> chimera (Ch) derived from G10 was heterozygous for the downstream *loxP* site (primers 1,2) and had a single amplicon (primers 6,7 *Neo Delete*) that was generated from the wild-type (WT) locus only because *Neo* was large. WT mice produce an amplicon corresponding to the endogenous loci with primer sets 1,2 or 6,7. *Wnt1*<sup>fl/+</sup>; *Flpe* mice had both *loxP* sites (primers 6,7 and 1,2) and the WT locus. (D-H) Control embryos had normal morphology, *Otx2* in the prosencephalon (pros), *Pax6* in the diencephalon (di) and anterior ventral hindbrain (v.Hb). *Wnt1* was in the caudal mes and *Fgf8* was in the isthmus. (I-M) *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup> mutants at E10.5 had a depleted mes/r1 (asterisk in I) and truncated v.Mes flexure (arrow in I). (J,K) *Otx2* in the pros, di, remaining mes/r1 (red arrow), and dorsal a.Hb (red arrowhead). *Pax6* overlapped with *Otx2* anteriorly, but not in the mes/r1 (red arrow) or a.Hb (red arrowhead). The *Otx2*<sup>+</sup>/*Pax6*<sup>-</sup> v.Mes (black arrows) was diminished in *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup> embryos. (L,M) *Wnt1* was not detected in remaining mes/r1 (red arrow), but was in lateral r1 (red arrowhead) whereas *Fgf8* was depleted in the a.Hb, ba, branchial arch; fl, forelimb; hl, hind limb; hyp, hypothalamus; ov, otic vesicle.

marker analysis. In controls, *Otx2*, *Pax6*, *Wnt1* and *Fgf8* were expressed in well-defined domains at E10.5, as previously described (Fig. 2E-H; supplementary material Fig. S4A-G). *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup> mutants at E10.5 had *Otx2* expression throughout the prosencephalon and diencephalon but not in the hypothalamus (Fig. 2J), which was similar to controls. *Otx2* was also expressed in the remaining putative mes (Fig. 2J, red arrow) and r1 (Fig. 2J, red arrowhead) based on morphology. *Pax6* delineated the diencephalon, but was not expressed in the remaining putative mes and r1 (Fig. 2K, red arrow and arrowhead, respectively) of conditional mutants. *Wnt1* was not detected in the putative mes, but a small domain was observed in lateral r1 (Fig. 2L, red arrow and arrowhead, respectively) of conditional mutants. The posterior hindbrain (Hb) of *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup> embryos was unaffected because it was outside of the *En1* domain (Fig. 2L). The presence of *Wnt1* in a small lateral r1 domain suggested that *En1*<sup>Cre</sup> did not mediate recombination in this territory or that *Wnt1* and *En1* did not overlap in this region. Finally, conditional deletion of *Wnt1* resulted in the absence of *Fgf8* expression in the isthmus (anterior r1) (Fig. 2M). The v.Mes was dysmorphic in mutants and the small portion of the mutant v.Mes flexure was *Otx2*<sup>+</sup>/*Pax6*<sup>-</sup> (Fig. 2J,K versus 2E,F, black arrows; supplementary material Fig. S4).

### Early requirement of *Wnt1* in the *En1* lineage

The molecular identity of conditional mutants suggested that some mes/r1 tissue persisted, which prompted us to assess the *En1* lineage in *Wnt1* conditional knockouts by coupling gene deletion and cumulative lineage marking. We compared *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/+</sup>; *R26*<sup>TdTomato</sup> control and *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>TdTomato</sup> mutant littermates at E12.5 ( $n \geq 3$  each genotype). Whole-mount analysis of controls showed the typical distribution of the *En1* lineage (Fig. 3A-C, red fluorescence). By contrast, *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>TdTomato</sup> mutant embryos had a substantial depletion of the *En1* lineage-derived mes (Fig. 3I-K). In addition, r1 was diminished and had only a small domain that was adjacent to axonal bundles connected to the trigeminal ganglia (Fig. 3I). Given that the v.Mes and ventral r1 (v.r1) are where MbDA and serotonin neurons are generated, respectively, we assessed *En1* lineage contribution with biomarkers of neural progenitors and differentiating neurons in these domains (as shown in Fig. 3C,K). The limits of the *En1* lineage delineated the anterior extent of the mes and the posterior limit of r1 in control embryos consistent with previous reports (Fig. 3D-H, arrowheads) (Zervas et al., 2004; Ellisor et al., 2009). OTX2, which plays a role in MbDA neuron development (Di Salvio et al., 2010; Omodei et al., 2008; Vernay et al., 2005) was distributed within the *En1*-derived domain





**Fig. 3. Early deletion of *Wnt1* depletes MbDA neurons.**

(A-H) *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/+</sup>; *R26*<sup>tdTomato</sup> embryo at E12.5. (A,B) *En1* lineage (red) contribution to the mes, r1, trigeminal ganglia (tg, asterisk) and craniofacial domain (cf). Axons traversed the tg and lateral a.Hb at the pontine flexure (yellow arrow). (C) Region of analysis (black box), v.Mes flexure (diamond) and *En1*-lineage (red shading). (D-H) Control sections immunolabeled for indicated markers (green) and the lineage tracer (dsred<sup>+</sup>, red). *En1*-derived cells were throughout the mes/r1 (delineated by arrowheads). (D) The mes/r1 boundary (arrows) coincided with posterior limit of OTX2. (E) LMX1a in the ventricular zone (vz, yellow bracket) up to the anterior limit of the v.Mes (white arrowheads). (F) MbDA neurons in the differentiated zone (dz, yellow bracket); inset shows TH<sup>+</sup> neurons at higher magnification. (G,H) 5-HT<sup>+</sup> neurons in v.r1. Numbers indicate rostral to caudal 5-HT<sup>+</sup> neurons as indicated in text. (I-P) *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>tdTomato</sup> mutants at E12.5 (I,J). *Wnt1* mutants were devoid of the mes (indicated by red arrow), but did have a small fused mes/r1 domain (mes/r1\*) and sparse projections at the pontine flexure (yellow arrow). (K) Region of analysis and *En1*-lineage (as described for C). (L-P) The *En1*-derived mes/r1 domain (red) was diminished, but a cohort of *En1* lineage-derived cells was present. The anterior boundary was irregular and dsRed<sup>+</sup> cells were in the di. (L,M) *Wnt1*-mutant/*En1*-lineage derived domain had OTX2<sup>+</sup> progenitors, but not LMX1a. (N) TH<sup>+</sup> MbDA neurons were depleted; inset shows sparse TH<sup>+</sup> neurons. (O) Anterior 5-HT<sup>+</sup> neurons were depleted whereas those at the edge of the *En1*-lineage domain (2) were diminished and those outside the *En1*-lineage domain (3) were unaffected. (P) Representative *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>tdTomato</sup> embryo with *En1*-lineage derived domain that was microdissected, sorted, and genotyped by PCR: *En1*-derived cells contained only the *Wnt1*<sup>Δ</sup> allele. cp, choroid plexus; di, diencephalon; fl, forelimb; hl, hind limb; m, mammillary body; pros, prosencephalon; pt, prepectum; rp, Rathke's pouch.

and had a sharp caudal limit at the mes/r1 boundary (Fig. 3D, arrows; supplementary material Fig. S5). LMX1a, which is a transcription factor proposed to be a determinant of MbDA neurons (Andersson et al., 2006), was robustly expressed in the ventricular zone and to a lesser extent in the differentiated zone adjacent to the v.Mes flexure with a caudal limit at the v.Mes/v.r1 boundary (Fig. 3E, arrows; supplementary material Fig. S5). LMX1a was also expressed in the ventral diencephalon (Fig. 3E). Tyrosine hydroxylase (TH)-expressing MbDA neurons were located in the differentiated zone of the *En1*-derived v.Mes of controls (Fig. 3F; supplementary material Fig. S5) whereas the cell bodies of 5-hydroxytryptamine (5-HT)-expressing serotonergic neurons were nested within the *En1*-derived r1 and had long anterior-coursing axons that traversed the v.Mes (Fig. 3G,H). *En1* lineage mapping provided insight into the nature of the tissue that remained in *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>tdTomato</sup> mutant embryos (Fig. 3L-O, dsred<sup>+</sup> domain located between arrowheads). The dorsal mes/r1 was severely depleted as evident by the close proximity of the prepectum to putative r1, although the ventral mes/r1 was less affected and had a prominent domain. The *En1*-derived mutant v.Mes contained OTX2<sup>+</sup> progenitors, but we did not observe LMX1a<sup>+</sup> progenitors throughout the medial-lateral extent of the v.Mes (Fig. 3L,M; supplementary material Fig. S5). Interestingly, LMX1a<sup>+</sup>

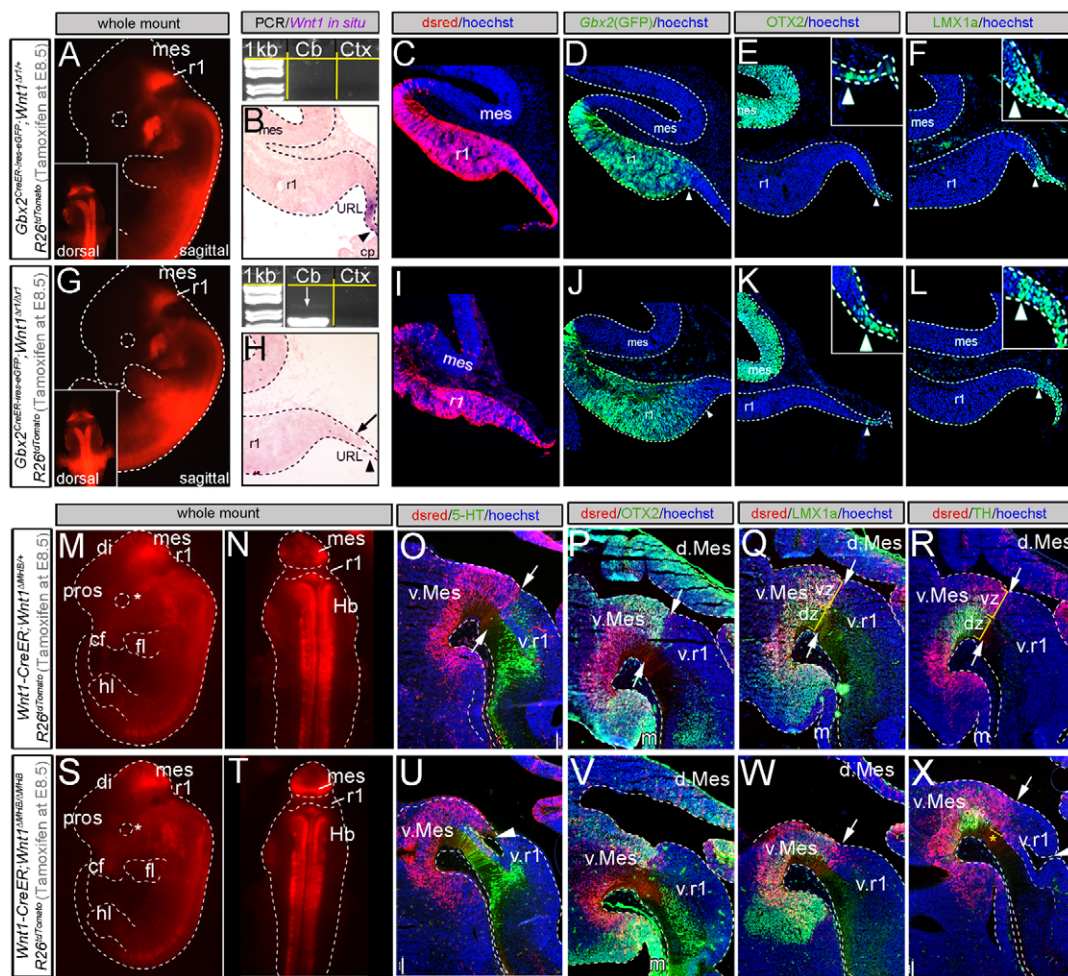
progenitors in the ventral diencephalon that were outside the *En1* domain were also diminished, suggesting a non-cell-autonomous effect resulting from the loss of *Wnt1* within the *En1*-derived domain (Fig. 3M). TH<sup>+</sup> MbDA neurons in conditional mutants were severely depleted with only a small, disorganized cohort remaining (Fig. 3N; supplementary material Fig. S5). The most rostral 5-HT<sup>+</sup> neurons (cluster 1) were absent in the remaining *En1*-derived ventral tissue (not shown), although more caudally positioned serotonergic neurons (clusters 2-4) were present (Fig. 3O; data not shown). We microdissected the mes/r1 from *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>tdTomato</sup> mutants and used fluorescence-activated cell sorting (FACS) to isolate *En1*-derived (dsred<sup>+</sup>) cells followed by PCR genotyping to detect the status of the *Wnt1* allele. The dsred<sup>+</sup> cells were *Wnt1*<sup>Δ</sup> and did not contain the floxed allele, which indicated that remaining tissue in *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup>; *R26*<sup>tdTomato</sup> embryos did not require *Wnt1* (Fig. 3P). Collectively, these findings showed that a domain of *En1* lineage-derived v.Mes tissue was established in the absence of *Wnt1*, but that both LMX1a-expressing progenitors and MbDA neurons have an early requirement for *Wnt1*. *En1*-lineage analysis also showed that neurons in the trigeminal ganglia of controls had processes that innervated whisker pads and the craniofacial region and processes that connected to the anterior hindbrain (a.Hb) by

way of the pontine flexure (supplementary material Fig. S6A,B, inset) consistent with previous studies (Ellisor et al., 2009). *En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>tdTomato</sup>* embryos were similar to controls and had trigeminal ganglia projections that innervated the craniofacial region and the pontine flexure (supplementary material Fig. S6C,D). However, analysis of whisker fields (location of vibrissae) and serotonergic innervation of the craniofacial region of conditional knockout embryos showed aberrantly patterned whisker fields innervated by serotonergic neurons and a severe truncation of the *En1*-derived lateral a.Hb (supplementary material Fig. S6C,D,G,H).

### ***Wnt1* in r1 is not required for Cb development and is transiently required for patterning**

We previously showed that late temporal epochs of *Wnt1*-expressing progenitors in the rhombic lip of r1 contribute to the Cb, consistent with late *Wnt1* expression in this domain (Hagan and Zervas, 2012).

Thus, r1 depletion in *En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/ΔMHB</sup>* embryos could be due to *Wnt1* function in the mes at the mes/r1 boundary or in the rhombic lip. We addressed this issue by deleting *Wnt1* in the rhombic lip using *Gbx2<sup>CreER-ires-eGFP</sup>* mice (Chen et al., 2009) plus our *Wnt1<sup>fl</sup>* allele and the *R26<sup>tdTomato</sup>* line to simultaneously mutate and track the recombination event (Fig. 4A-L). Pregnant dams were treated with tamoxifen at E8.5 to mediate recombination in r1; *Wnt1* was effectively deleted based on molecular analysis to detect the deleted allele and *in situ* hybridization to show loss of *Wnt1* in r1 (Fig. 4A,B,G,H). We evaluated *Gbx2* lineage contribution (*dsred<sup>+</sup>*) *Gbx2*(GFP), OTX2 and LMX1a expression in r1, which were unaffected by the loss of *Wnt1* (Fig. 4C-F,I-L). Thus, the deletion of r1 was due to *Wnt1* function in the mes and not in the rhombic lip. We next tested the hypothesis that *Wnt1* continues to be required for patterning the mes/r1 and for MbDA neuron development using *Wnt1-CreER* mice (Zervas et al., 2004) coupled with tamoxifen to mediate recombination in *Wnt1*-expressing



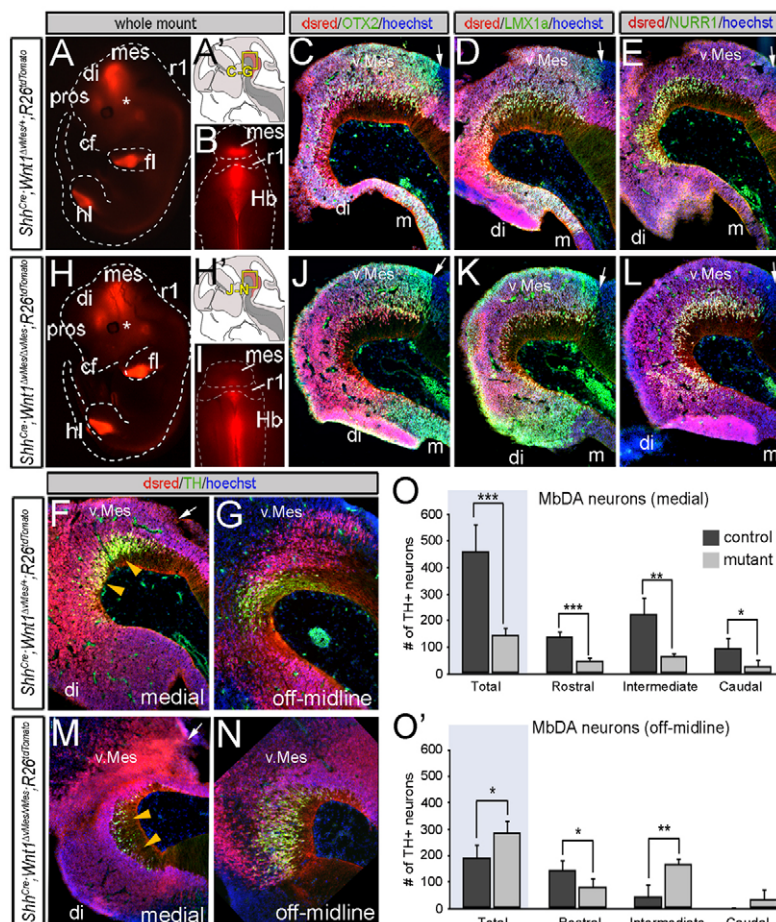
**Fig. 4. Temporal requirement of *Wnt1* for patterning the Mb and Cb.** (A-F) Control E12.5 *Gbx2<sup>CreER-ires-eGFP</sup>;Wnt1<sup>Δr1</sup>;R26<sup>tdTomato</sup>* embryos received tamoxifen at E8.5 and were analyzed for the *Gbx2* lineage, *Wnt1*, *Gbx2*(GFP), OTX2 and LMX1a. Arrowheads indicate limit of marker expression. (G-L) E12.5 *Gbx2<sup>CreER-ires-eGFP</sup>;Wnt1<sup>Δr1/Δr1</sup>;R26<sup>tdTomato</sup>* mutants that received tamoxifen at E8.5 had a loss of *Wnt1* in the upper rhombic lip (URL). The *Gbx2* lineage, *Gbx2*(GFP), OTX2 and LMX1a were unaffected. Arrowheads indicate limit of marker expression. (M-X) *Wnt1-CreER;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>tdTomato</sup>* controls that received tamoxifen at E8.5 and analyzed at E12.5 showed the *Wnt1* lineage (red) in the mes, trigeminal ganglia (asterisk) and craniofacial domain (cf). (O-R) Controls labeled for markers (green) and the lineage tracer (*dsred<sup>+</sup>*, red) showed *Wnt1*-derived cells throughout the v.Mes, but not in r1. The *Wnt1* lineage abutted 5-HT<sup>+</sup> neurons in v.r1 and expressed OTX2 and LMX1a in the vz. *Wnt1*-lineage derived TH<sup>+</sup> neurons in the dz. (S-X) *Wnt1-CreER;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>tdTomato</sup>* mutants had a smaller v.Mes versus controls although the *Wnt1* lineage (red) was unaffected. 5-HT<sup>+</sup> neurons were unaffected. *Wnt1*-derived cells expressed OTX2 and LMX1a, but caudal MbDA neurons were depleted (asterisk, bracket in X). cp, choroid plexus; Ctx, cerebral cortex; di, diencephalon; dz, differentiated zone; fl, forelimb; hl, hindlimb; m, mammillary body; pros, prosencephalon; vz, ventricular zone.



progenitors at E8.5 (Fig. 4M-X). *Wnt1*-derived cells (red fluorescence) were similarly distributed in the mes, posterior Hb, and spinal cord in both *Wnt1-CreER;Wnt1<sup>ΔMHB/+</sup>;R26<sup>TdTomato</sup>* and *Wnt1-CreER;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>TdTomato</sup>* littermates at E12.5 (Fig. 4M,N,S,T). In control embryos, *Wnt1*-derived cells in the v.Mes abutted the 5-HT<sup>+</sup> neurons in the a.Hb and co-expressed OTX2 and LMX1a (Fig. 4O-Q). TH<sup>+</sup> MbDA neurons in *Wnt1-CreER;Wnt1<sup>ΔMHB/+</sup>;R26<sup>TdTomato</sup>* embryos were located in the differentiating zone of the v.Mes with a posterior limit at the mes/r1 boundary (Fig. 4R). The v.Mes in *Wnt1-CreER;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>TdTomato</sup>* embryos was smaller than that in controls and contained an abnormal notch, but was otherwise patterned normally based on the morphology of the v.Mes (Fig. 4U-X). The mutant *Wnt1* lineage marked at E8.5 expressed OTX2 and LMX1a and was distributed similar to controls (Fig. 4V,W). However, the deletion of *Wnt1* at E8.5 resulted in depletion of TH<sup>+</sup> neurons in the medial v.Mes, but not of 5-HT<sup>+</sup> neurons in v.r1 (Fig. 4U,X). Interestingly, we also observed an increase in TH<sup>+</sup> neurons in the off-midline plane of *Wnt1* conditional knockout mutants (data not shown). These phenotypes were observed in two out of four embryos with the variability probably arising from the mosaic nature of recombination with this transgenic line. We also administered tamoxifen to *Wnt1-CreER;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>TdTomato</sup>* embryos at E10.5, which did not result in any overt phenotype in four out of four embryos (supplementary material Fig. S7). These findings indicated that the patterning role of *Wnt1* was early, transient, and restricted to the mes. In addition, a subset of MbDA neurons continued to require *Wnt1* for their development between E8.5 and E10.5.

### *Wnt1* has a continued role in MbDA neuron development and regulates cell cycle exit

We used the *Shh<sup>Cre</sup>* line (Harfe et al., 2004; Hayes et al., 2011; Tang et al., 2010; Tang et al., 2009) to address the continued role of *Wnt1* because *Shh<sup>Cre</sup>* mediates robust and cumulative recombination in the v.Mes beginning at E9.0-9.5 (Harfe et al., 2004; Hayes et al., 2011), which is later than with our *En1<sup>Cre</sup>* mice. To validate that *Shh<sup>Cre</sup>* was appropriate to delete *Wnt1* we used FACS to sort v.Mes cells from *Wnt1-Venus* mice (Brown et al., 2011; Ellisor et al., 2012; Hagan and Zervas, 2012) and performed qRT-PCR that showed that *Wnt1*(GFP)-expressing MbDA neuron progenitors also express *Shh* (supplementary material Fig. S8). Because *Shh<sup>Cre</sup>* mediates recombination in the v.Mes, we operationally define *Wnt1<sup>ΔvMes</sup>* as the recombined allele for this experiment (Fig. 5). The *Shh* lineage in E12.5 *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/+</sup>;R26<sup>TdTomato</sup>* embryos analyzed by whole-mount fluorescence and on immunolabeled sagittal sections had a typical distribution of OTX2<sup>+</sup>, LMX1a<sup>+</sup> and NURR1<sup>+</sup> progenitors and early differentiating TH<sup>+</sup> neurons (Fig. 5A-G). *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* littermates had a normal distribution of the *Shh* lineage and OTX2<sup>+</sup>, LMX1a<sup>+</sup> and NURR1<sup>+</sup> progenitors (Fig. 5H-L). However, we observed a depletion of medial MbDA neurons and an expansion of more laterally positioned MbDA neurons in mutants (Fig. 5F,G,M,N). Quantification of medial populations revealed TH<sup>+</sup> counts of 462±103 (mean±s.d.) versus 147±27 from controls and conditional mutants, respectively ( $n \geq 4$  each genotype), which was a significant decrease in MbDA neurons (Fig. 5O;  $P=0.0006$ ). By contrast, off-midline TH<sup>+</sup> counts were 192±51 and 288±45 from control and mutants, respectively, which was a corresponding significant



**Fig. 5. Late temporal requirement of *Wnt1* for MbDA neuron development.** (A-G) *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/+</sup>;R26<sup>TdTomato</sup>* controls at E12.5 showed the *Shh* lineage (red) in the v.Mes. (C-G) Sections immunolabeled for indicated markers (green) and the lineage tracer (dsred<sup>+</sup>, red) showed that the *Shh* lineage expressed OTX2, LMX1a and NURR1. (F,G) *Shh* lineage-derived medial and off-midline TH<sup>+</sup> neurons. Orange arrowheads indicate TH<sup>+</sup> neurons. White arrows indicate MHB. Box in A' indicates the region shown in C-G. (H-N) *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* mutants did not have morphological abnormalities. (J-L) The v.Mes morphology was unaffected and *Shh*-derived cells expressed OTX2, LMX1a and NURR1<sup>+</sup>, similar to controls. (M,N) Medial TH<sup>+</sup> MbDA neurons (arrowheads) were severely reduced whereas off-midline TH<sup>+</sup> neurons were expanded in mutants. White arrows indicate the MHB. (O,O') Quantification of TH<sup>+</sup> MbDA neurons in medial and off-midline planes including rostral to caudal positions from the v.Mes of control and conditional mutants (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Error bars represent s.d. Asterisk in A,H indicates trigeminal ganglia. cf, craniofacial; di, diencephalon; fl, forelimb; hl, hindlimb; m, mammillary body; pros, prosencephalon.

increase of off-midline MbDA neurons (Fig. 5O';  $P=0.020$ ). These changes were observed at rostral, intermediate and caudal positions in the v.Mes (Fig. 5O,O').

MbDA neurons differentiate such that early versus late born neurons occupy more lateral or medial positions, respectively (Kawano et al., 1995; Bayer et al., 1995; Bye et al., 2012). To test whether the MbDA neuron phenotype in *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* embryos was caused by alterations in cell cycle exit we administered EdU at E11.5 and analyzed embryos 24 hours later by confocal microscopy and quantitative analysis to detect Hoechst (nuclear counter stain), dsred (*Shh* lineage), TH (terminally differentiated MbDA neurons), EdU (birthdating) and Ki67 (proliferation) (Fig. 6; supplementary material Fig. S9). *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* controls had  $160\pm33$  (mean $\pm$ s.d.) *Shh* lineage-derived medial MbDA neurons that differentiated at E11.5 (TH<sup>+</sup>/EdU<sup>+</sup>/Ki67<sup>-</sup>) (Fig. 6A-F,Y). By contrast, *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* mutants had  $38\pm14$  medial MbDA neurons that differentiated at E11.5 (TH<sup>+</sup>/EdU<sup>+</sup>/Ki67<sup>-</sup>), which was a significant fourfold reduction (Fig. 6G-L,Y;  $P=0.0002$ ). Control embryos had negligible  $3\pm3$  off-midline TH<sup>+</sup>/EdU<sup>+</sup>/Ki67<sup>-</sup> neurons that differentiated at E11.5 (Fig. 6M-R,Y') whereas mutant embryos had  $38\pm26$ , which was a significant increase of MbDA neurons that had differentiated at E11.5 (Fig. 6S-X,Y';  $P=0.0200$ ). Both control and mutant embryos had similar counts of proliferating progenitors (Ki67<sup>+</sup>). However, mutants had significantly more Ki67<sup>+</sup> progenitors compared with controls ( $420\pm152$  versus  $155\pm139$ , respectively;  $P=0.0330$ ) in the differentiating zone in medial planes. Finally, we compared the ratio of E11.5 birthdated MbDA neurons (TH<sup>+</sup>/EdU<sup>+</sup>/Ki67<sup>-</sup>) to all birthdated cells (EdU<sup>+</sup>/Ki67<sup>-</sup>) to test whether the phenotype we observed was specific to MbDA neurons or the result of global deficits in cell cycle exit. *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* embryos had a TH<sup>+</sup>/EdU<sup>+</sup>/Ki67<sup>-</sup> to EdU<sup>+</sup>/Ki67<sup>-</sup> ratio of  $0.18\pm0.05$  (Fig. 6Z, dark gray bars) whereas *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>TdTomato</sup>* embryos had a ratio of  $0.05\pm0.02$  (Fig. 6Z, light gray bars), which was a significant reduction ( $P=0.005$ ). By contrast, the ratio off-midline was  $0.01\pm0.01$  for controls (Fig. 6Z', dark gray bars) and  $0.08\pm0.07$  for mutants (Fig. 6Z', light gray bars), which was a significant increase ( $P=0.01$ ). The rostral-to-caudal distribution showed similar results (Fig. 6Z,Z'). Thus, the conditional deletion of *Wnt1* resulted in a loss of medial MbDA neurons as well as a smaller fraction of medial cells that differentiated into MbDA neurons and a concomitant larger fraction of ectopic laterally positioned MbDA neurons (Fig. 6AA).

## DISCUSSION

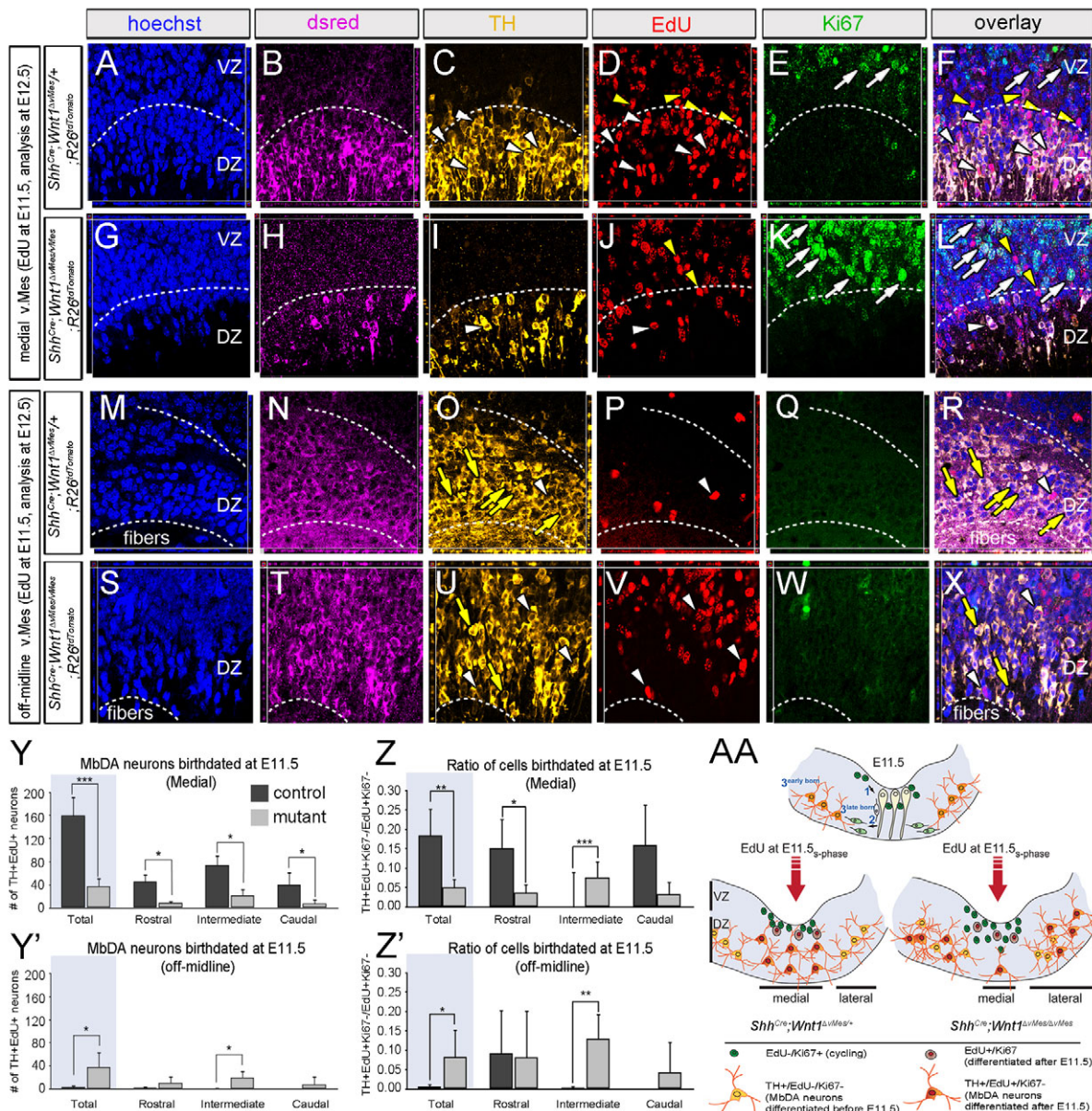
*Wnt1* is important for Mb and a.Hb development (McMahon and Bradley, 1990; McMahon et al., 1992), but the role of *Wnt1* at specific stages of embryonic development or within specific genetic lineages has been elusive because *Wnt1* mutant mice that were previously available for analysis were derived by neomycin disruption (null alleles) or were caused by a spontaneous mutation (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990; Thomas et al., 1991). We circumvented these limitations by generating a *Wnt1<sup>f</sup>* allele, which we used in spatial and temporal deletion experiments in combination with genetic lineage tracing. We show that *Wnt1* patterns the d.Mes and v.Mes, and patterns r1 during an early and brief window (~12 hours between 0-1 somites and 6-8 somites). By tracing the *En1* lineage in *Wnt1* conditional mutants, we show that a large portion of *En1*-derived tissue is depleted at E12.5. However, cells derived from the *En1* lineage are present in mutants with a more substantial allotment

in v.Mes and v.r1 compared with dorsal tissue. The presence of an *En1*-derived *Wnt1*-mutant domain indicates that a cohort of cells derived from the *En1* lineage does not require *Wnt1*. Thus, the Mb is derived from a heterogeneous pool of progenitors and is partitioned by complex molecular identities and genetic lineages. The deletion of *Wnt1* in the rhombic lip of r1 did not phenocopy the truncation of r1, which provides definitive experimental evidence that *Wnt1* in the mes is functionally required for r1 development, probably by regulating the organizer molecule FGF8.

OTX2<sup>+</sup> progenitors persist in *En1*-derived *Wnt1*-mutant v.Mes tissue, suggesting that signaling by *Wnt1* does not induce or maintain *Otx2* at least in a subset of v.Mes cells. This is in contrast to viral-mediated transduction of *Wnt1* in ES cells, which increases *Otx2* mRNA levels (Chung et al., 2009). Thus, *Wnt1* regulation of *Otx2* might be context dependent. The remaining *En1*-derived *Wnt1*-mutant domain is devoid of LMX1a<sup>+</sup> cells, suggesting that *Wnt1*-dependent progenitors (which may also be the LMX1a-expressing population) are unable to expand in the absence of *Wnt1* or that a cohort of mutant progenitors may not have been induced resulting in a smaller initial progenitor pool. At the same time, a *Wnt1*-independent population may expand with many of those cells still expressing OTX2. We show that *Wnt1* requirement for LMX1<sup>+</sup> progenitors is transient and occurs between the 1-somite and the 6- to 8-somite stages. This finding complements previous observations that *Wnt1*(GFP) is expressed more broadly and prior to LMX1a in the v.Mes (Brown et al., 2011). It has been proposed that *Lmx1a* functions as a determinant of MbDA neurons (Andersson et al., 2006); interestingly, WNT1 uses the canonical  $\beta$ -catenin pathway to regulate *Lmx1a* expression and, conversely, LMX1a binds directly to the promoter of *Wnt1* during MbDA neuron differentiation (Chung et al., 2009). Thus, an alternative explanation for the phenotype of the early deletion of *Wnt1* is that WNT1 initiates *Lmx1a* expression followed by a short pulse of WNT signaling and *Lmx1a*-*Wnt1* cross-regulatory reinforcement. Later, an uncoupling of *Lmx1a* and *Wnt1* might occur in MbDA neuron progenitors, as suggested by results of our conditional deletion of *Wnt1* with *Wnt1-CreER* or *Shh<sup>Cre</sup>* lines. This dynamic regulation is consistent with how WNT1 temporally regulates oscillations of gene expression and genetic pathway interactions *in vitro* (Wexler et al., 2011). We also show that even though a v.Mes domain persists with early *Wnt1* deletion, TH<sup>+</sup> neurons are not generated, which is consistent with the absence of LMX1a. Interestingly, medial MbDA neurons have a continued requirement of *Wnt1* for their development that occurs between E8.5 and E10.5. Given that LMX1a<sup>+</sup> progenitors are unaffected by the late deletion of *Wnt1*, we suggest that later-born medial MbDA neurons develop independently of LMX1a. The presence of *Wnt1*-dependent/*Lmx1a*-dependent (early) and *Wnt1*-dependent/*Lmx1a*-independent (later) progenitors is consistent with only a subset of MbDA neurons being depleted in *Lmx1a* mutant mice (Ono et al., 2007). Collectively, our findings show that *Wnt1* is a key upstream regulator in MbDA neuron development and that MbDA neurons are derived from progenitors that express *Wnt1* early and continue to express *Wnt1*.

SHH and FGF8 function cooperatively to induce MbDA neurons during a short developmental window and together enhance the efficiency of producing MbDA-like neurons from ES cells and induced pluripotent stem cells (iPSCs) (Ye et al., 1998; Lee et al., 2000; Soldner et al., 2009). *Wnt1*-expressing v.Mes progenitors express components consistent with these signaling pathways and also express *Fzd9* (supplementary material Fig. S8). Thus, we show that MbDA neuron progenitors are poised to respond to SHH, FGF8 and WNT1 signaling. Previous studies have shown that canonical





**Fig. 6. Late conditional *Wnt1* deletion alters cell cycle exit of MbDA neurons *in vivo*.** (A–X) EdU was administered at E11.5 and embryos were analyzed at E12.5. (A–F, M–R) *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/+</sup>;R26<sup>tdTomato</sup>* controls. (G–L, S–X) *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>tdTomato</sup>* mutants. Medial sections (A–L) and off-midline sections (M–X) are shown. Sections were co-labeled for Hoechst (A,G,M,S), dsred (B,H,N,T), TH (C,I,O,U), EdU (D,J,P,V) and Ki67 (E,K,Q,W). Overlay is shown in F,L,R,X. Medial domains of *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>tdTomato</sup>* mutants had more cells in the active cell cycle at E11.5 (Ki67<sup>+</sup>, white arrows) compared with controls. Fewer cells exited the cell cycle and differentiated into MbDA neurons (EdU<sup>+</sup>/TH<sup>+</sup>/Ki67<sup>+</sup>, white arrowheads) in mutants versus controls. Yellow arrowheads indicate EdU<sup>+</sup>/TH<sup>+</sup>/Ki67<sup>+</sup> cells. Off-midline domains of *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>tdTomato</sup>* mutants showed increased MbDA neurons birthdated at E11.5 (EdU<sup>+</sup>/TH<sup>+</sup>/Ki67<sup>+</sup>, white arrowheads). MbDA neurons born outside the range of EdU kinetics were EdU<sup>+</sup>/TH<sup>+</sup>/Ki67<sup>+</sup> (yellow arrows). Dashed line indicates boundary between ventricular zone (VZ) and differentiated zone (DZ). (Y–Z') Quantitative assessment of *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/+</sup>;R26<sup>tdTomato</sup>* controls (dark gray bars) and *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>;R26<sup>tdTomato</sup>* mutants (light gray bars) at E12.5. (Y,Y') Total, rostral, intermediate and caudal MbDA neurons birthdated at E11.5 (TH+EdU+) in medial and off-midline planes. (Z,Z') Ratio of MbDA neurons born at E11.5 (TH+EdU+/Ki67-) to the total number of cells born at E11.5 (EdU+/Ki67-). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. Error bars represent s.d. (AA) Illustration of cell cycle exit data. At E11.5, Ki67<sup>+</sup> cells (green cells) proliferate in the VZ, subsequently migrate along radial glia (beige cells), and differentiate into TH<sup>+</sup> MbDA neurons (gold neurons); the early born MbDA neurons are located more lateral than the later born MbDA neurons. The addition of EdU at E11.5 tracks differentiating MbDA neurons (red shading, gold neurons). In *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/+</sup>* embryos, TH+/EdU+/Ki67- MbDA neurons were primarily located in a medial location whereas TH+/EdU+/Ki67- MbDA neurons were located laterally. The conditional deletion of *Wnt1* in *Shh<sup>Cre</sup>;Wnt1<sup>ΔvMes/ΔvMes</sup>* embryos disrupted the timing of cell cycle exit, which caused the depletion of medial MbDA neurons and a concomitant increase of ectopic, laterally positioned TH+/EdU+/Ki67- MbDA neurons.

WNT signaling can antagonize *Shh* expression (Joksimovic et al., 2009). However, our early deletion of *Wnt1* shows that *Shh* expression is not increased and suggests that WNT1 does not drive

canonical WNT-mediated antagonism of *Shh* in the v.Mes and that the phenotype we observe is not due to changes in *Shh*. It is possible that the early deletion of *Wnt1* and the near-complete loss of MbDA

neurons were secondary to the changes in *Fgf8*. However, we do not believe that this is the case because, unlike our *En1<sup>Cre</sup>;Wnt1<sup>fl/fl</sup>* conditional mutants, which have a near-complete depletion of MbDA neurons at E12.5, the deletion of *Fgf8* (at the same time and using the same *Cre* line) in *En1<sup>Cre</sup>;Fgf8<sup>fl/fl</sup>* embryos results in a normal complement of TH<sup>+</sup> neurons at E12.5 (Ellisor et al., 2012). Interestingly, ES cells treated with SHH and FGF8 express *Wnt1* prior to becoming MbDA-like neurons (Lee et al., 2000) (M.Z., unpublished results) and *Wnt1* is important for SHH- and FGF8-mediated ectopic induction of MbDA neurons (Prakash and Wurst, 2006). Therefore, the role of *Wnt1* in MbDA neuron development is independent of *Fgf8* and *Shh*, although future experiments are needed to address how the convergence of FGF8, SHH and WNT1 signaling shapes MbDA neuron progenitors.

Deletion of *Wnt1* in the *Shh* lineage (*Shh<sup>Cre</sup>;Wnt1<sup>fl/fl</sup>*) reveals that OTX2, LMX1a and NURR1 are unaffected, which is similar to observations in *Shh<sup>Cre</sup>;β-catenin<sup>fl/fl</sup>* mice described by Tang et al. (Tang et al., 2009) using the same *Shh<sup>Cre</sup>* line. We show that the cell cycle of only MbDA neurons are affected in our conditional *Wnt1* mutants as reflected in the decreased ratio of MbDA neurons/all cells born. By contrast, conditional β-catenin mutants have a decrease in the birthdating of all cells, including MbDA neurons. These findings suggest that disrupting β-catenin-dependent WNT signaling causes a more global disruption of cells going through S phase than does the deletion of WNT1 ligand alone. The reduction of MbDA neurons born at E11.5 is similar in conditional β-catenin and our conditional *Wnt1* mutants (~50% decrease in E11.5-born MbDA neurons in both mutants), suggesting that cell cycle progression and neurogenesis of MbDA neurons is largely mediated by WNT1 via canonical signaling. Interestingly, disruption of the cell cycle is linked to the depletion of differentiated MbDA neurons (as shown by decreased total numbers of TH<sup>+</sup> neurons), and *Wnt1* deletion in the v.Mes results in MbDA neurons that are displaced and improperly positioned, which may be due to changes in cell migration. Although this may shape MbDA neuron subtype identity, the early phenotype we observe does not directly address whether *Wnt1* and cell cycle alteration affects ventral tegmental area (VTA) or substantia nigra (SNc) identity in adults, which will be important to resolve in future studies. In summary, our analyses reveal that *Wnt1* is an important regulatory component that controls cell cycle exit and resolve the spatial and temporal roles for *Wnt1* in specific developmental processes and in MbDA neurons during embryogenesis.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Author contributions

*Wnt1<sup>fl</sup>* allele work was carried out by J.Y. and D.E.; ES cell work by E.P. and D.E.; *En1<sup>Cre</sup>*-mediated deletion of *Wnt1<sup>fl</sup>* and qRT-PCR by D.E. and M.Z.; *Gbx2<sup>CreER-ires-eGFP</sup>* experiments by N.H.; and *Wnt1-CreER* and *Shh<sup>Cre</sup>* experiments by A.B. The paper was written by M.Z., D.E. and A.B.

#### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.080630/-DC1>

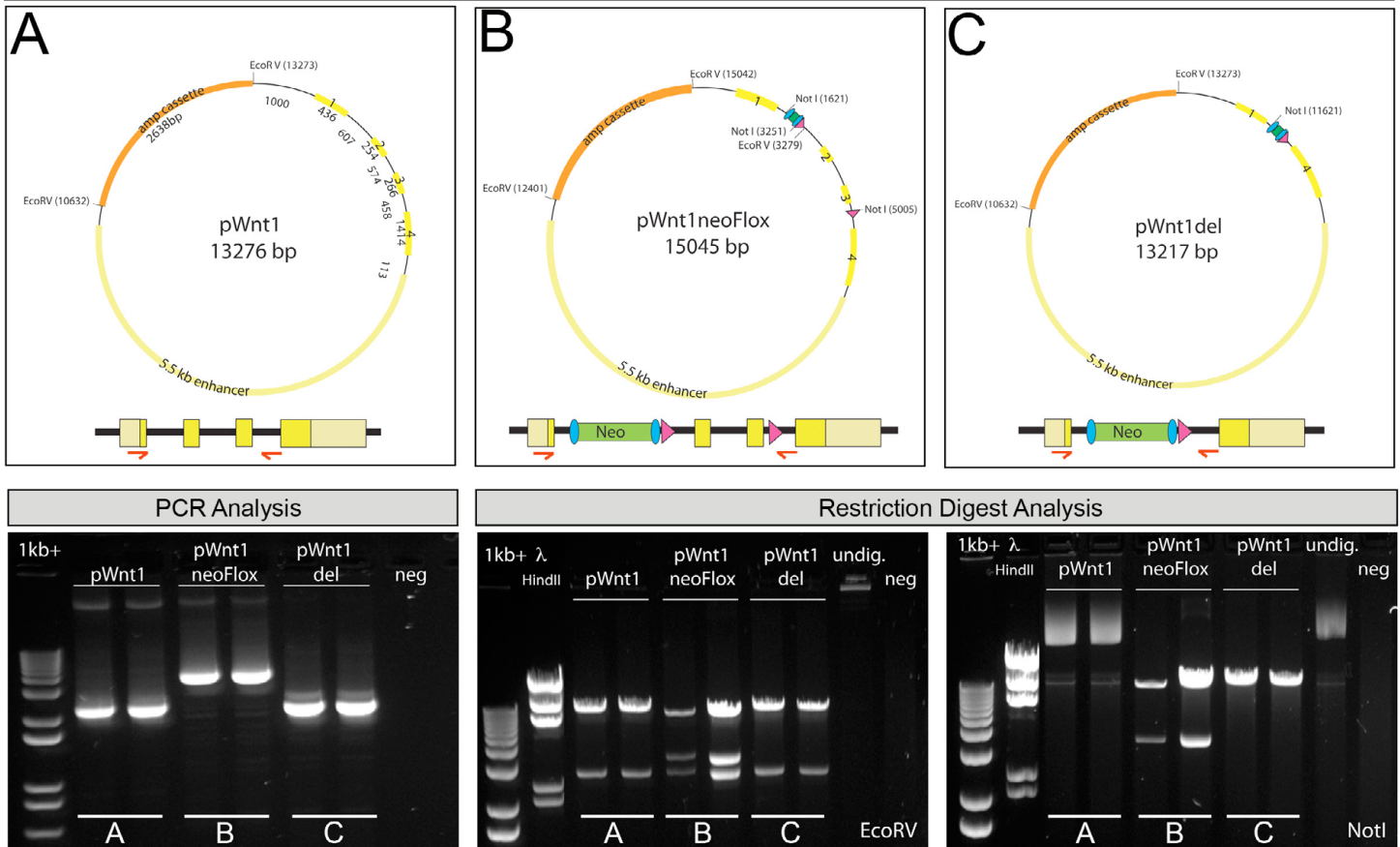
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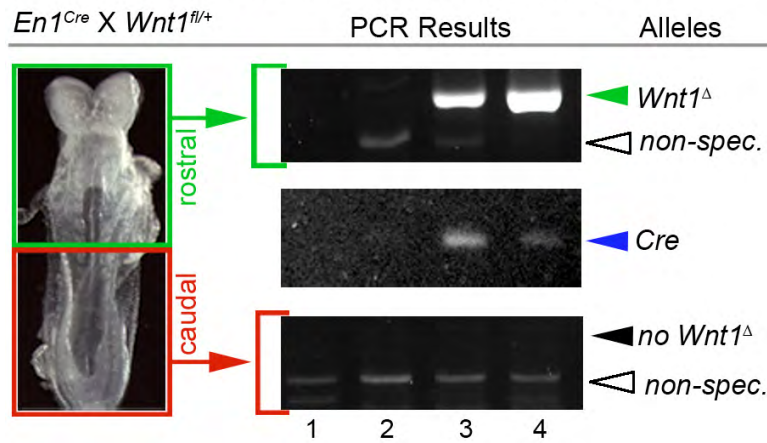
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## Recombineering (Plasmid Design)

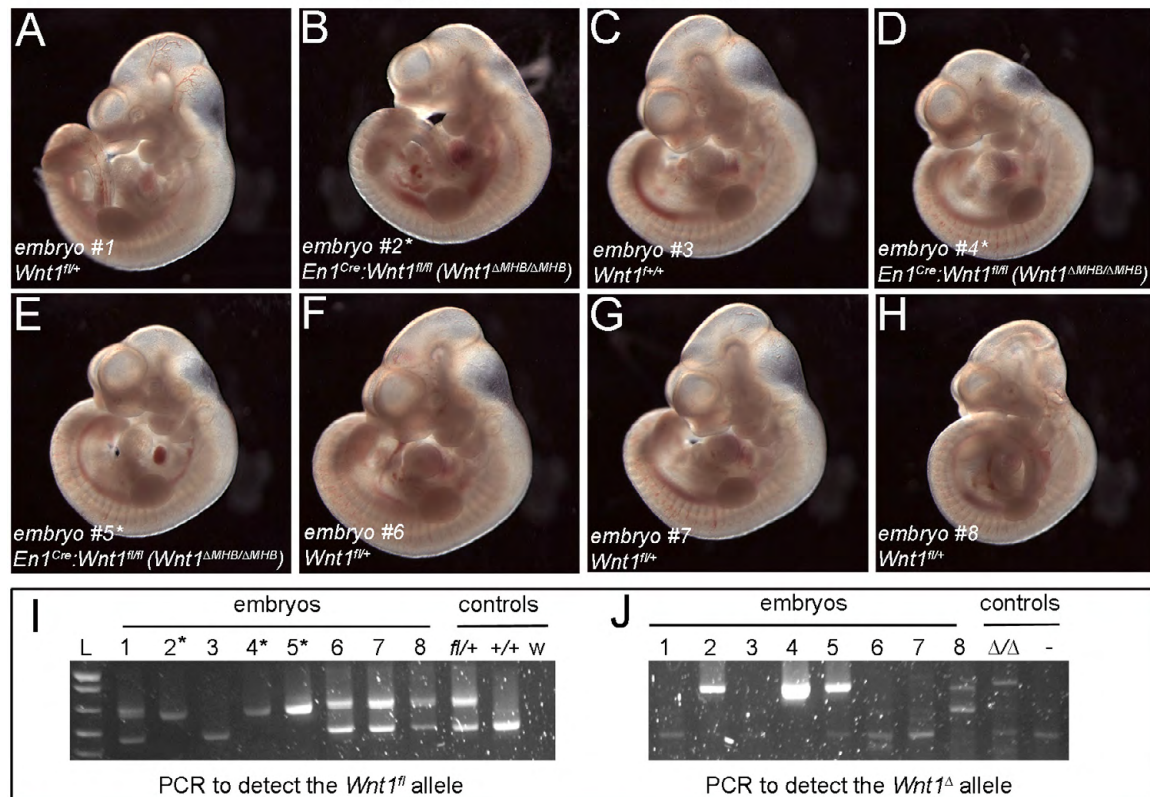


**Fig. S1. BAC recombineering and generation of the conditional *Wnt1<sup>fl</sup>* allele.** (A-C) Schematics of constructs used to generate the *Wnt1<sup>fl</sup>* allele. Linear drawings show regions modified by recombineering and assessed by PCR analysis. (A) The entire 10.6-kb *Wnt1* gene including sequence upstream of the promoter and downstream of the 5.5-kb enhancer, was captured from a BAC library by homologous recombination using GeneBridges recombineering kits with 50-bp homologous arms to generate the plasmid pWnt1. pWnt1 also contains an *EcoRV* restriction site, an *ori* site and an ampicillin-resistant gene (*amp*) used for subcloning. (B) Recombineering steps included inserting a *loxP* site between exons 3 and 4. An *FRT*-*PGK*-*gb2*-*Neo*-*FRT*-*loxP* cassette was also inserted between exons 1 and 2 to generate pWnt1<sup>neoFloX</sup>. (C) The recombination potential of the modified (pWnt1<sup>neoFloX</sup>) construct was tested by expressing the plasmid in DH10B *E. coli* cells. Cells were then electroporated with *pKS*-*Cre* to delete Exons 2 and 3, which were located between *loxP* sites in the same orientation. Gels: A, B and C in gels refer to plasmids in corresponding lettered panels. Left-hand gel: PCR analysis showing two examples of pWnt1 that produced a single amplicon using primers positioned in exon 1 and in intronic sequence between exons 3/4 (A). The same primers produced a larger amplicon in two examples of pWnt1<sup>neoFloX</sup> (B) because of the modifications to pWnt1. Electroporation of *pKS*-*Cre* into DH10B cells produced pWnt1<sup>del</sup> by the deletion of exons 2/3 (C). Restriction digest with *EcoRV* (middle gel) or *NotI* (right gel) validated recombineering-based modifications. pWnt1<sup>neoFloX</sup> (B) had an additional *EcoRV* and *NotI* site not present in pWnt1 (A) and that were lost after *Cre*-mediated deletion in pWnt1<sup>del</sup> (C).

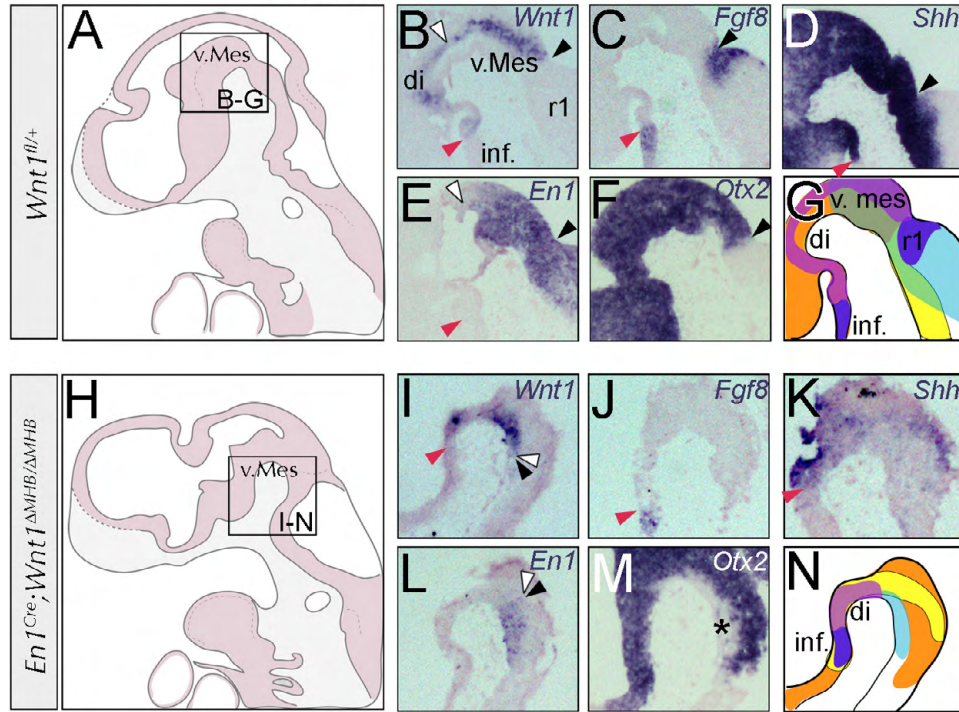




**Fig. S2. Validation of the *Wnt1* conditional knockout allele in vivo.** *Wnt1<sup>fl/+</sup>* males were bred with *En1<sup>Cre</sup>* females in timed matings and embryos were dissected at the 6-8 somite stage. The rostral and caudal half of embryos were isolated, placed in separate PCR tubes, and lysed. Representative PCR analysis revealed that in the absence of *En1<sup>Cre</sup>*, the *Wnt1<sup>fl</sup>* allele was unaltered (lanes 1, 2). By contrast, embryos that inherited *En1<sup>Cre</sup>* and the floxed allele produced *Wnt1<sup>Δ</sup>* (lanes 3, 4). Importantly, the *Wnt1<sup>Δ</sup>* allele was detected in the rostral half of the embryo because *En1*, and therefore *Cre*, was expressed only in the mes/r1 (anterior tissue) and not caudally at this stage (Li et al., 2002).

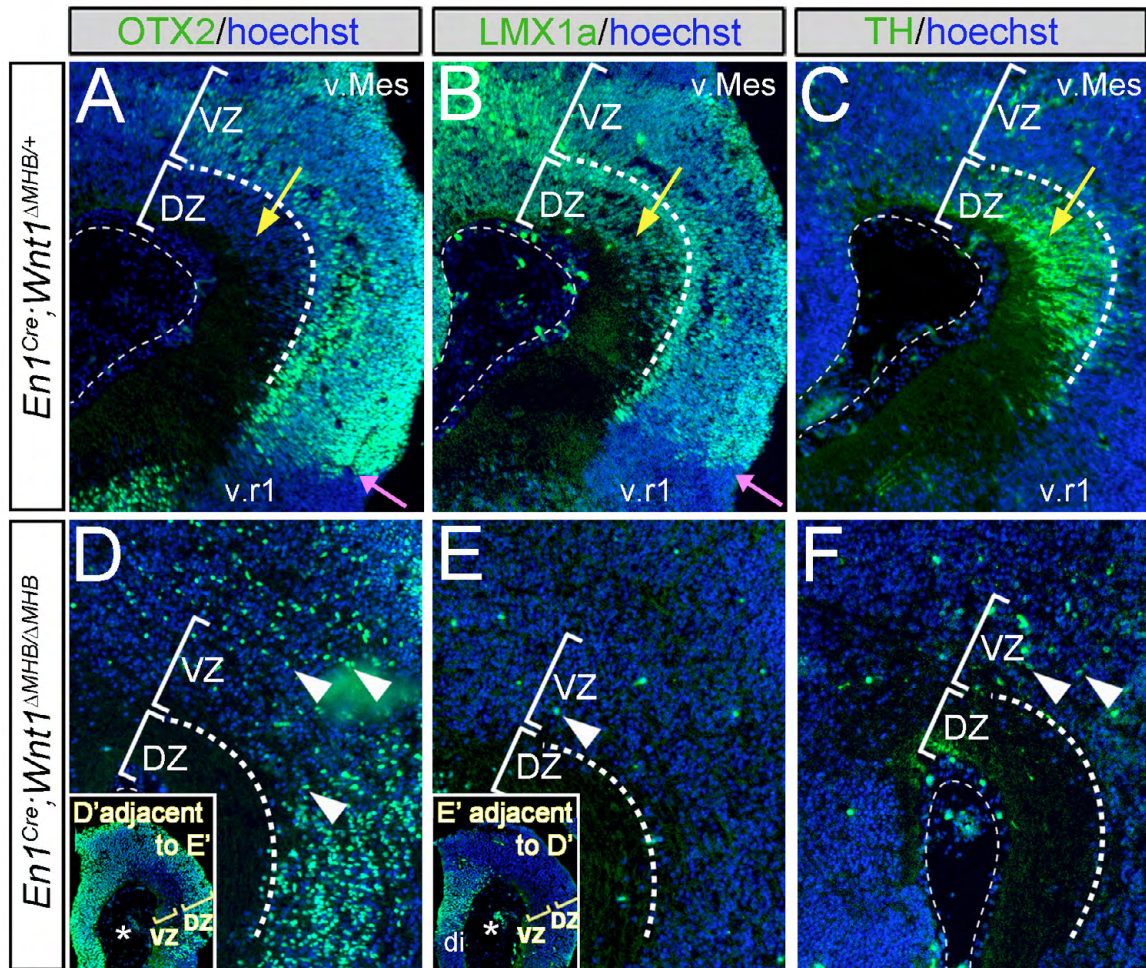


**Fig. S3. Conditional deletion of *Wnt1* results in mes and r1 deletion.** Breeding of *En1<sup>Cre</sup>;Wnt1<sup>fl/+</sup>* with *Wnt1<sup>fl/+</sup>* mice produced putative conditional mutant littermates. (A,C,F,G,H) Morphologically normal embryos at E10.5 had an intact mes and r1. (B,D,E) Three littermates (*En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/ΔMHB</sup>*) had significantly depleted mes/r1 domains. (I) PCR detected the *Wnt1<sup>fl</sup>* allele from DNA obtained from a tail biopsy: Embryos 2\*,4\*,5\* were homozygous for the floxed allele (*Wnt1<sup>fl/fl</sup>*). Embryo 3 was wildtype for *Wnt1* and embryos 1,6,7,8 were heterozygotes (*Wnt1<sup>fl/+</sup>*). Embryos 2\*,4\*,5\* were also *Cre<sup>+</sup>* by PCR (not shown). (J) PCR to detect the deleted allele showed that embryos 2\*,4\*,5\* had both copies of the *Wnt1<sup>fl</sup>* alleles converted to *Wnt1<sup>ΔMHB/ΔMHB</sup>* alleles. Controls were heterozygous adult tail DNA or water substituted for DNA template (w).

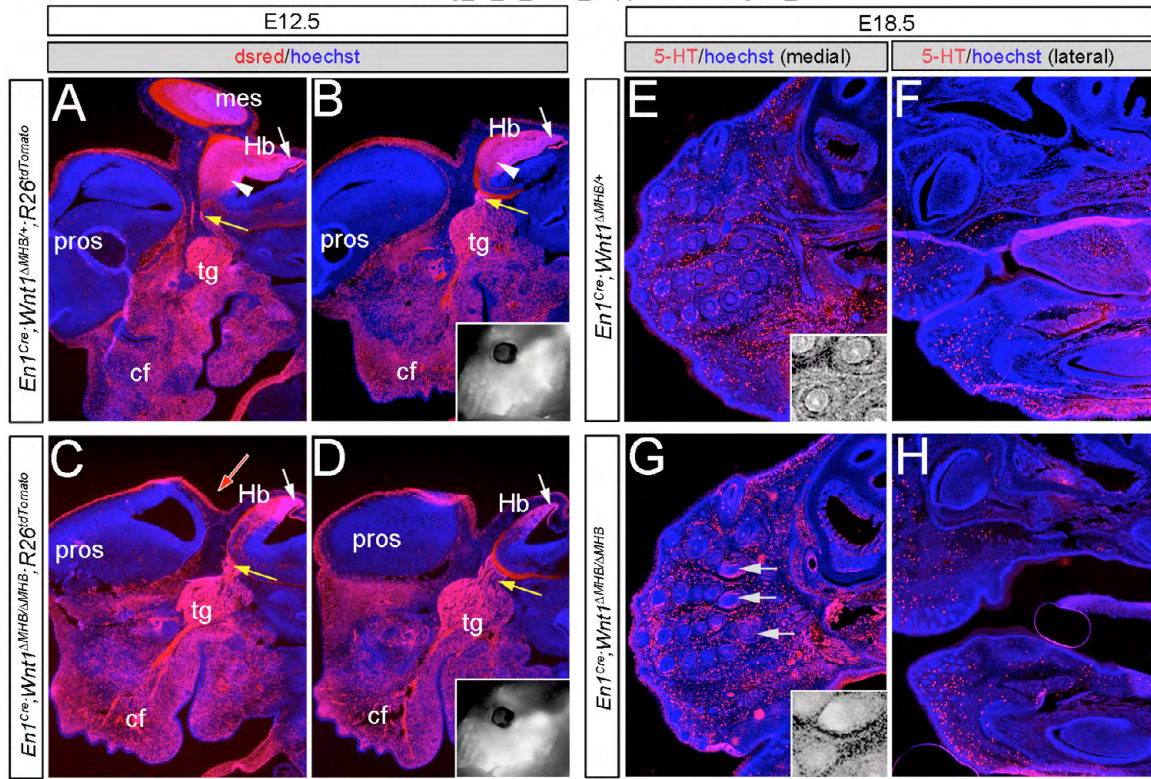


**Fig. S4. Conditional deletion of *Wnt1* disrupted the molecular identity of the v.Mes.** (A-G) The v.Mes and v.r1 of E10.5 control embryos were analyzed by *in situ* hybridization. (A) Regions of analysis. (B) *Wnt1* was in the v.Mes (between white and black arrowheads) and the di (between red and white arrowheads). (C) *Fgf8* was localized to the organizer that was identified by a clear notch (black arrowhead) and in the infundibulum (inf). (D) *Shh* in the v.Mes and v.r1 up to the limit of the inf. (red arrowhead). (E) *En1* in the v.Mes and v.r1. (F) *Otx2* had a sharp line of demarcation at the mes/r1 boundary (black arrowhead). (G) Summary schematic of genes in control embryos: *Wnt1* (purple), *Shh* (yellow), *Fgf8* (blue), *En1* (aqua), *Otx2* (orange); the green domain indicates overlap between *Shh* and *En1* and the olive domain indicates *Shh*, *En1*, *Otx2*. (H-N) *En1*<sup>Cre</sup>; *Wnt1*<sup>ΔMHB/ΔMHB</sup> embryos at E10.5. (H) Regions of analysis in conditional mutants. (I) The v.Mes flexure had a small *Wnt1* domain in the presumptive di. Significant depletion of *Wnt1* occurred in the *En1*-expressing domain (between white and black arrowheads). However, we cannot rule out the possibility that some cells that initially expressed *Wnt1* prior to the commencement of *En1*<sup>Cre</sup> did not undergo recombination. (J) *Fgf8* was depleted in the organizer, but not in the inf. (K) A small *Shh* domain persisted in the flexure, but was less intensely labeled than in controls. (L) *En1* was in a small domain caudal to the remaining *Wnt1* domain. (M) *Otx2* in the ventral flexure exhibited a salt and pepper expression pattern in the caudal region. (N) Summary schematic of gene expression in mutants (Colors are the same as in G).





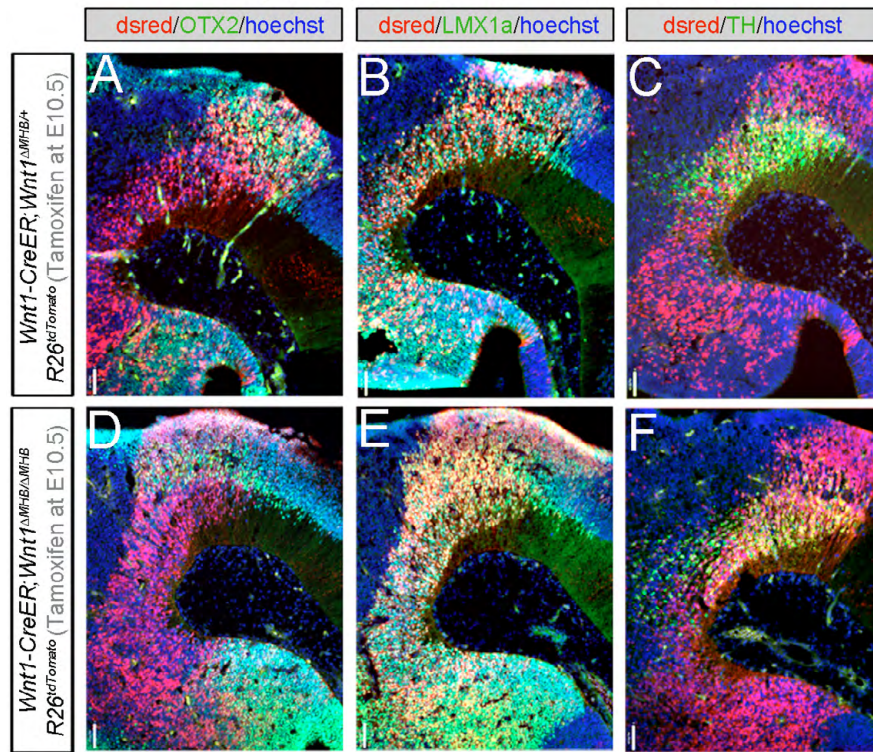
**Fig. S5. Comparison of markers in embryos with early deletion of *Wnt1*.** (A-F) Sagittal sections of control *En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/+</sup>* (A-C) and mutant *En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/ΔMHB</sup>* (D-F) embryos at E12.5 were immunolabeled for OTX2, LMX1a or TH (markers are in green) and counterstained with Hoechst (blue) as indicated. (A,B) Controls had OTX2<sup>+</sup> cells localized to the ventricular zone (VZ) and LMX1a<sup>+</sup> cells in both the VZ and differentiating zone (DZ). (D,E) Mutants had OTX2<sup>+</sup> cells scattered in the VZ. LMX1a was not detected in the v.Mes. (C) TH<sup>+</sup> dopamine neurons were distributed in the DZ of controls. (D) Only a few TH<sup>+</sup> neurons were observed in mutants. Yellow arrows indicate the v.Mes flexure, arrowheads indicate examples of cells in mutants, and purple arrows indicate the v.Mes/v.r1 lineage boundary. Panels A-C and D-F show 12 μm-thick sections that are directly adjacent to each other and are approximately 100 μm from the sections in the manuscript (See Fig. 3D-F,L-N). Insets in D',E': Sagittal sections (12 μm thick) directly adjacent to each other and approximately 100 μm from the *En1<sup>Cre</sup>;Wnt1<sup>ΔMHB/ΔMHB</sup>* mutant sections shown in panels D and E (described above). OTX2 (D'), but not LMX1a (E') was detected in the v.Mes. LMX1a<sup>+</sup> cells were observed in the diencephalon (di). TH<sup>+</sup> dopamine neurons were not observed in this plane and are not shown. Asterisks indicate a cluster of blood vessels confirming the close proximity of sections. Collectively, these sections validate that the phenotype was not confined to one medial-lateral region but occurred throughout the medial-lateral extent of the v.Mes.



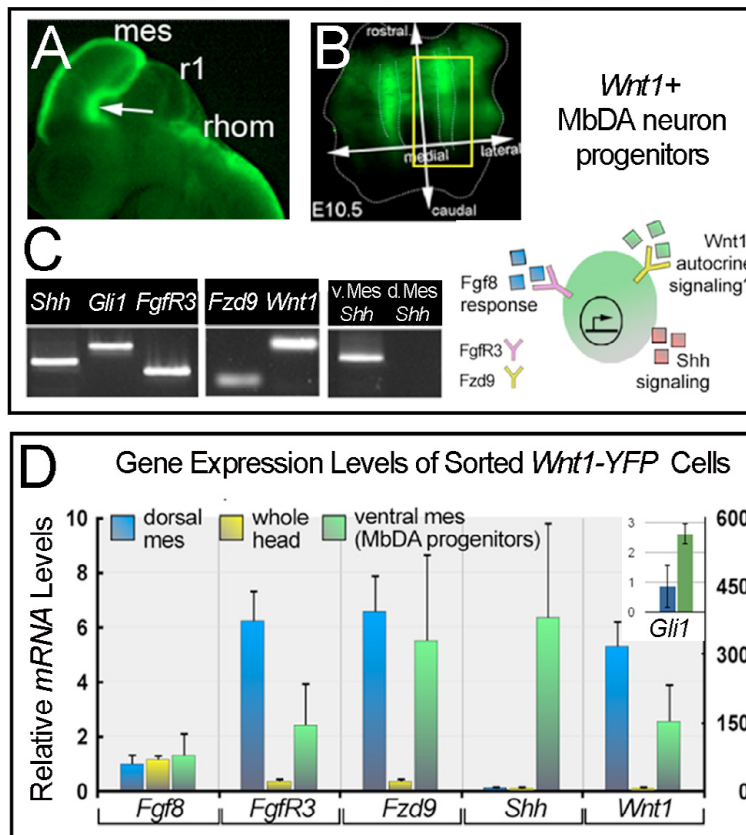
**Fig. S6. Neural crest-derived structures assessed after conditional gene deletion and GFM. (A,B)**

$En1^{Cre}; Wnt1^{\Delta MHB/+}; R26^{tdTomato}$  control embryo at E12.5 showing neural tube and neural crest structures derived from the *En1*-lineage (dsred<sup>+</sup>, red) in lateral (A) and off-midline (B) sections. The mes, trigeminal ganglia (tg), craniofacial (cf) region and a.Hb, are indicated. White arrowheads and white arrows show, respectively, the anterior and posterior portions of the a.Hb. The prosencephalon (pros) and diencephalon (di), which were not derived from the *En1* lineage, were detected by Hoechst counterstain (blue). Inset shows wholemount of the tg and cf domain. (C,D)  $En1^{Cre}; Wnt1^{\Delta MHB/+}; R26^{tdTomato}$  mutant embryo at E12.5. (C) Lateral section showing depleted mes (red arrow indicates absence of tissue). In addition, the ventral a.Hb was depleted leaving only a small posterior domain in the a.Hb (white arrow). Axons at the pontine flexure (yellow arrows) and the tg and cf domains were intact. (D) Off-midline sections from *Wnt1* conditional mutants also showed truncated ventral a.Hb but normal pontine flexure, tg and cf. (E,F) Medial (E) and lateral (F) sections from  $En1^{Cre}; Wnt1^{\Delta MHB/+}; R26^{tdTomato}$  embryos at E18.5 immunolabeled to detect 5-HT+ projections (red), which heavily projected to cf tissue and innervated between whisker fields. Whisker fields had a typical fingerprint whirl pattern delineated with hoechst counterstaining (blue). Black and white inset shows hoechst staining. (G,H) Medial (G) and lateral (H) sections from  $En1^{Cre}; Wnt1^{\Delta MHB/\Delta MHB}; R26^{tdTomato}$  embryos showed a general reduction of projections in the lower jaw cf tissue. Whisker fields as seen with hoechst counterstaining (blue) were not patterned properly (arrows). Black and white inset shows hoechst staining and poorly patterned whisker fields.



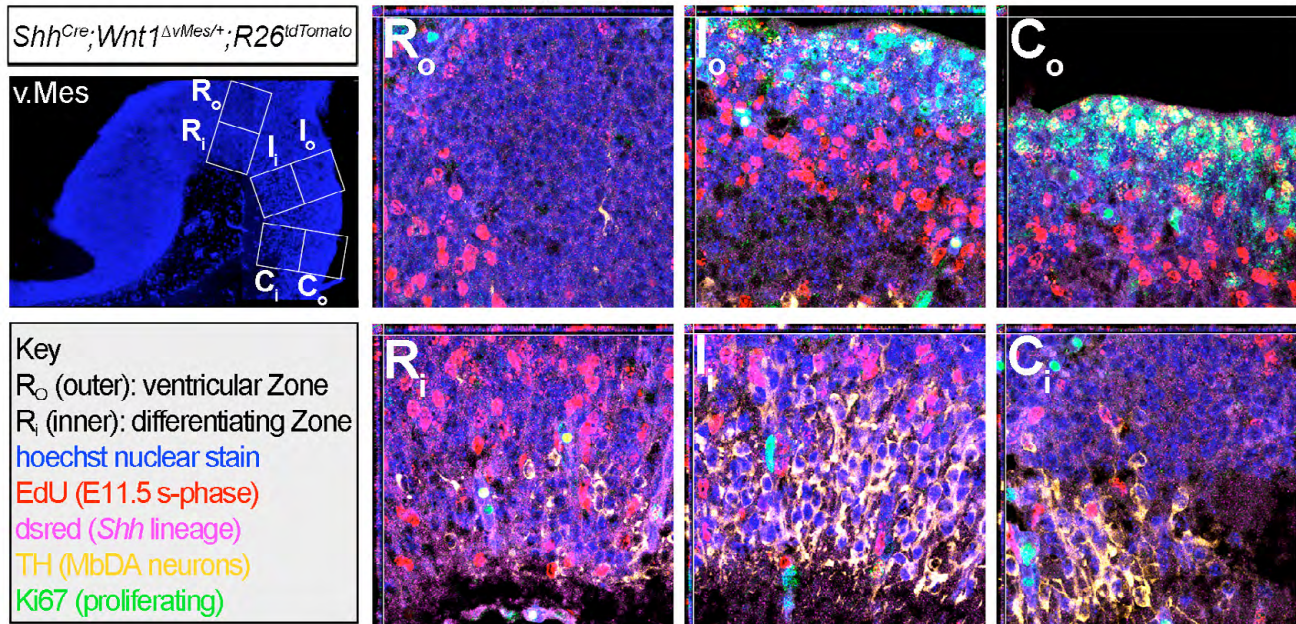


**Fig. S7. *Wnt1* is temporally required for MbDA neuron development.** Tamoxifen was given at E10.5 and embryos were analyzed at E12.5. (A-C) *Wnt1-CreER;Wnt1<sup>ΔMHB/+</sup>;R26<sup>tdTomato</sup>* controls. Sections immunolabeled with antibodies recognizing indicated markers (green) and the lineage tracer (dsred<sup>+</sup>, red) showed *Wnt1*-derived cells distributed in the v.Mes. (A,B) *Wnt1* lineage cells expressed OTX2 and LMX1a. (C) *Wnt1*-lineage derived TH<sup>+</sup> MbDA neurons in the dz. The caudal limit of the MbDA neurons aligned with posterior *Wnt1* lineage-derived cells. (D-F) *Wnt1-CreER;Wnt1<sup>ΔMHB/ΔMHB</sup>;R26<sup>tdTomato</sup>* mutants. Section analysis showed that the v.Mes and *Wnt1*-derived cells expressed OTX2 (D), LMX1a (E) and TH (F) similar to control embryos. Scale bars: 40 μm.



**Fig. S8. Molecular characterization of the *Wnt1* lineage during development.** (A) *Wnt1*-Venus embryo at E10.5 showing the location of putative MbDA neuron progenitors in the v.Mes (arrow). (B) The v.Mes was microdissected (Brown et al., 2009) to clearly identify the bilateral v.Mes YFP<sup>+</sup> progenitors, which were then isolated by FACS. The yellow box indicates the area of interest on one side. (C) Gels showing single PCR products after qRT-PCR from YFP<sup>+</sup> cells to detect the presence of FGF8, SHH and WNT signaling components involved in MbDA neuron development (shown in schematic). (D) Relative mRNA levels from FACS sorted YFP<sup>+</sup> d.Mes and v.Mes progenitors and whole head after qRT-PCR. The v.Mes progenitors (green bars) expressed *FgfR3*, *Fzd9*, *Shh*, *Wnt1* and *Gli1* (inset). The right y-axis pertains to *Wnt1* and *Shh* expression. Error bars represent s.d. See supplementary material Table S1 for primers used for qRT-PCR.





**Fig. S9. Sampling scheme for quantitative cell cycle analysis.** Representative image of the v.Mes. The boxes indicate where 40× z-series stacks were collected to sub-sample the tissue. The counting frames were organized in the following manner to analyze specific domains in the v.Mes along the rostral-to-caudal axis. Six counting frames were used and are designated as follows: rostral-outer (R<sub>o</sub>), rostral-inner (R<sub>i</sub>), intermediate-outer (I<sub>o</sub>), intermediate-inner (I<sub>i</sub>), caudal-outer (C<sub>o</sub>) and caudal-inner (C<sub>i</sub>). Cells were counted as described in Materials and methods.

**Table S1. Sequence of homology used to generate oligonucleotides for PCR.** Oligonucleotides used to generate linear vectors for modifying *Wnt1* by homologous recombination using GeneBridges recombineering kits. The construct for generating the *Wnt1* conditional knockout was generated with the recombineering system from Gene Bridges and the specific oligonucleotides indicated above to generate linear vectors for modifying *Wnt1*. First, the entire *Wnt1* gene (10.6 kb) including the promoter region, all coding exons, and the 5.5 kb downstream enhancer, was subcloned from BACs RP23-12305 and RP23-349E22 (CHORI) using a BAC subcloning kit (Gene Bridges: K003). Specifically, we used a linear vector generated by PCR using oligonucleotides that contained 50 bp sequence homologous to a 5' region upstream of the promoter or 3' region downstream from the 5.5 kb enhancer of *Wnt1* (Subcloning). Next, a *loxP-FRT-PGK-gb2-neo-FRT* cassette (Gene Bridges: A005) was inserted between the third and fourth exons (*LoxP*) followed by positive selection and *Flp* mediated removal of the *Neo* cassette leaving a single *loxP* site behind. A second *FRT-PGK-gb2-neo-FRT-loxP* cassette (Gene Bridges: A004) was inserted between the first and second exons, which was used for screening ES cells (*LoxP-FRT*).

**Table S1. Sequence of homology used to generate oligonucleotides for PCR**

<b>Subcloning</b>	AGATGTGCTAGTCATGAAGATGAACCGGGCTTGTTTCTCTGGCAACTAGG
	TGTCCTCCGGCTCTTCATTTACCTAGTTCTTGGTCAAAAGAGATGTCTAG
<b><i>LoxP</i></b>	CTGATGACAGAACAGAAAGAGATTAGCAGGCTATCAACACGTGGGATGTAT
	CGCCAGCAAGTCACTTTTATCTTTCAAAAGTGTGCCATGGAGCCATCTCA
<b><i>LoxP-FRT</i></b>	GTGACTTCACATCCAGGGTGCTCACACCTAGAACTAGCTCTGCTGAAGTG
	GGTCTCTGAGCCTGGTGTATCTGGGCTTCTGCATGCCAATGATGTGCCCC



**Table S2. PCR primers used to validate modifications**

Primer	Sequence	Comments
1	5'-CCA ATG GCA GGG GAG ATG TAA G-3'	Downstream <i>loxP</i>
2	5'-GAG GGA AGA AAA GGT CAA AGC-3'	
3	5'-CAA CAA AAC CAA AAG GAG CCA G-3'	5' end of construct (with Primer 5)
4	5'-TGG CTC GCA CAC ATT CCA CAT C-3'	Screening ES cells for <i>Neo</i>
5	5'-ATG GTT TAG TTC CTC ACC TTG TCG-3'	
6	5'-ACA GCA ACC ACA GTC GTC-3'	Upstream <i>loxP</i> once <i>Neo</i> is deleted
7	5'-TTT GGT TCA AGA CCT AAG GGA CAC-3'	
8	5'-GAA GCC CGT ACT TCA CAA CC-3'	Validates <i>Amp</i>
9	5'-CGA CAC GGA AAT GTT GAA TAC-3'	

Table S3. Sequence of primers used for qRT-PCR		
<b><i>Fgf8</i></b>	Fwd: 5'-CATTGTGGAGACCGATACTTTTGG-3'	122 bp amplicon
	Rev: 5'-AGTCCTTGCCTTTGCCGTTG-3'	
<b><i>FgfR3</i></b>	Fwd: 5'-ATCCTCGGGAGATGACGAAGAC-3'	314 bp amplicon
	Rev: 5'-GGATGCTGCCAAACTTGTTCTC-3'	
<b><i>Fzd9</i></b>	Fwd: 5'-CATCATGGAGCAATTCAATTTTCG-3'	51 bp amplicon
	Rev: 5'-CGGGCACAGTCGAGTGAGT-3'	
<b><i>Wnt1</i></b>	Fwd: 5'-GGCCGAGATTCTGTGGACT-3'	
	Rev: 5'-GCATCTCAGAGAACACGGTCG-3'	
<b><i>Shh</i></b>	Fwd: 5'- GGAAGATCACAAGAAACTCCGAAC-3'	354 bp amplicon
	Rev: 5'- GGATGCGAGCTTTGGATTCATAG-3'	
<b><i>Gli1</i></b>	Fwd: 5'- TGTTGTGGGAGGGAAGAAAC -3'	102 bp amplicon
	Rev: 5'- TGGCAGGGCTCTGACTAACT -3'	