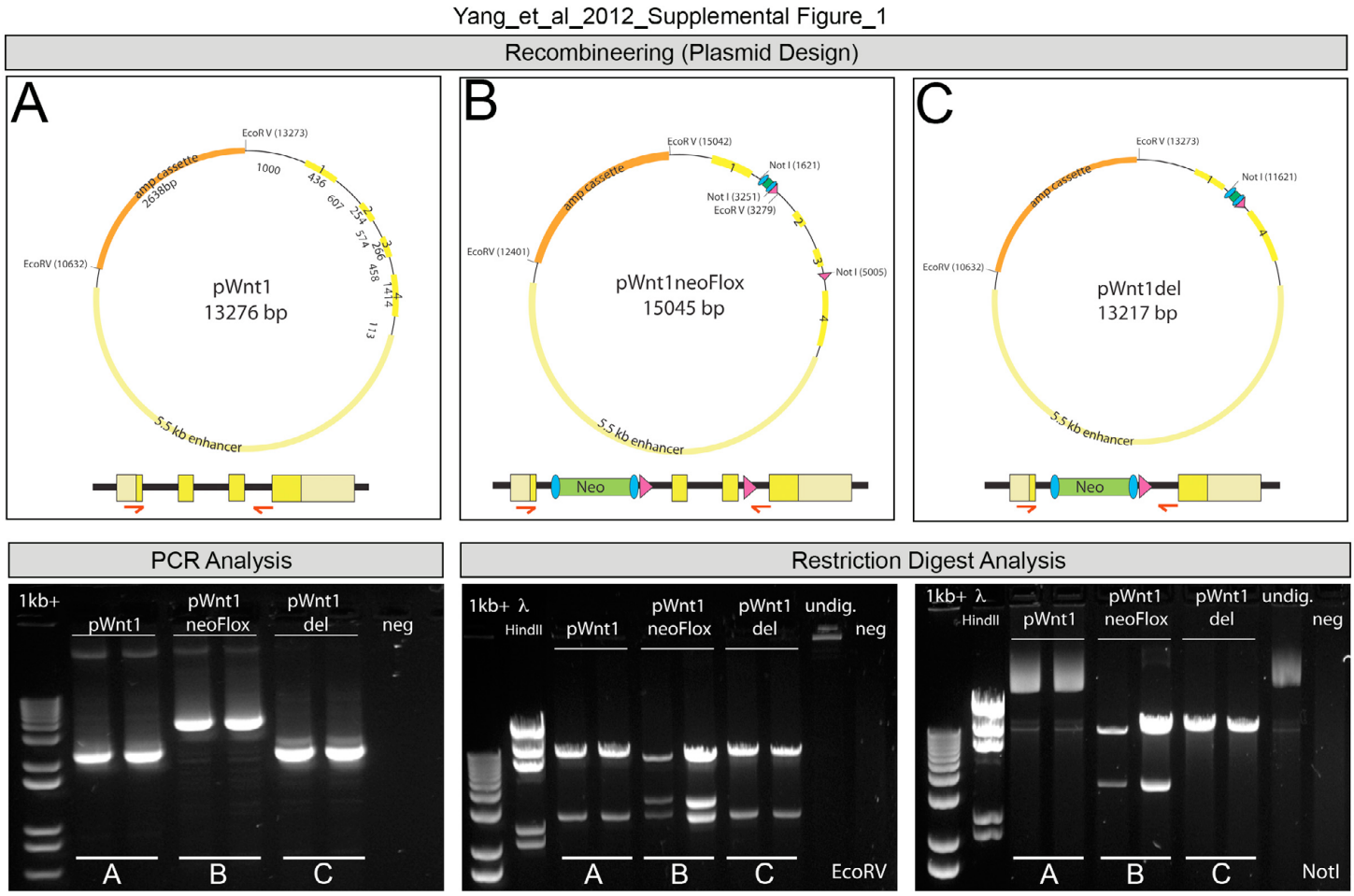

Fig. S1. BAC recombineering and generation of the conditional *Wnt1^{fl}* allele. (**A-C**) Schematics of constructs used to generate the *Wnt1^{fl}* allele. Linear drawings show regions modified by recombineering and assessed by PCR analysis. (A) The entire 10.6-kb *Wnt1* gene including sequence upstream 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 p*Wnt1*. *pWnt1* also contains an *Eco*RV 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^{neoFlox}*. (C) The recombination potential of the modified (*pWnt1^{neoFlox}*) 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^{neoflox}* (B) because of the modifications to *pWnt1*. Electroporation of *pKS-Cre* into DH10B cells produced *pWnt1^{del}* by the deletion of exons 2/3 (C). Restriction digest with *Eco*RV (middle gel) or *Not*I (right gel) validated recombineering-based modifications. *pWnt1^{neoflox}* (B) had an additional *Eco*RV and *Not*I site not present in *pWnt1* (A) and that were lost after Cre-mediated deletion in *pWnt1^{del}* (C).

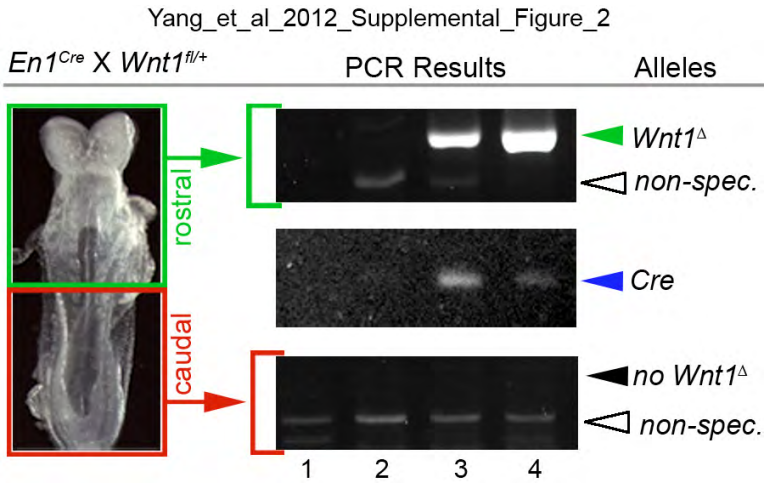

Fig. S2. Validation of the *Wnt1* conditional knockout allele in vivo. *Wnt1*$^{fl/+}$ males were bred with *En1*^{Cre} females in timed matings and embryos were dissected at the 6-8 somite stage. The rostral and caudal half of embryos were isolated, placed in separate PCR tubes, and lysed. Representative PCR analysis revealed that in the absence of *En1*^{Cre,} the *Wnt1*^{fl} allele was unaltered (lanes 1, 2). By contrast, embryos that inherited *En1*^{Cre} (*Cre*) and the floxed allele produced *Wnt1*$^{\Delta}$ (lanes 3, 4). Importantly, the *Wnt1*$^{\Delta}$ 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).

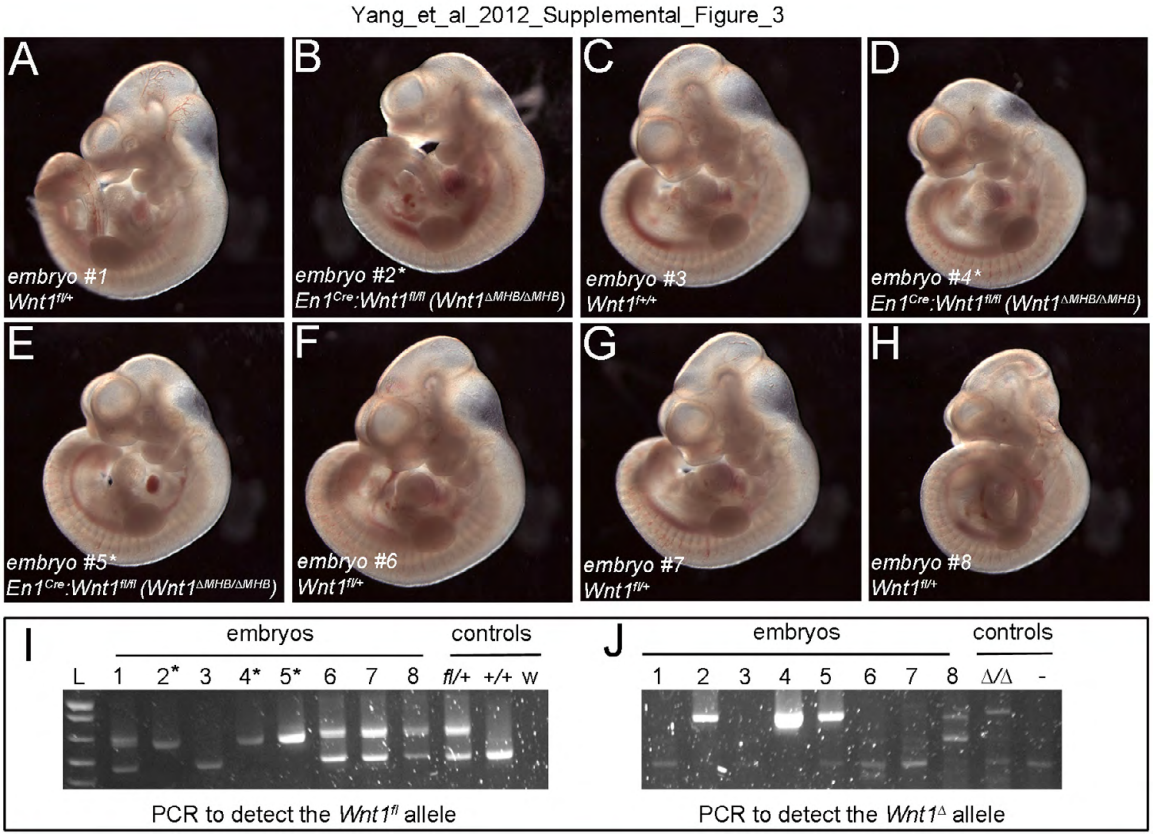

Fig. S3. Conditional deletion of *Wnt1* results in mes and r1 deletion. Breeding of *En1*^{Cre};*Wnt1*$^{fl/+}$ with *Wnt1*$^{fl/+}$ mice produced putative conditional mutant littermates. (**A**,**C**,**F**,**G**,**H**) Morphologically normal embryos at E10.5 had an intact mes and r1. (**B**,**D**,**E**)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

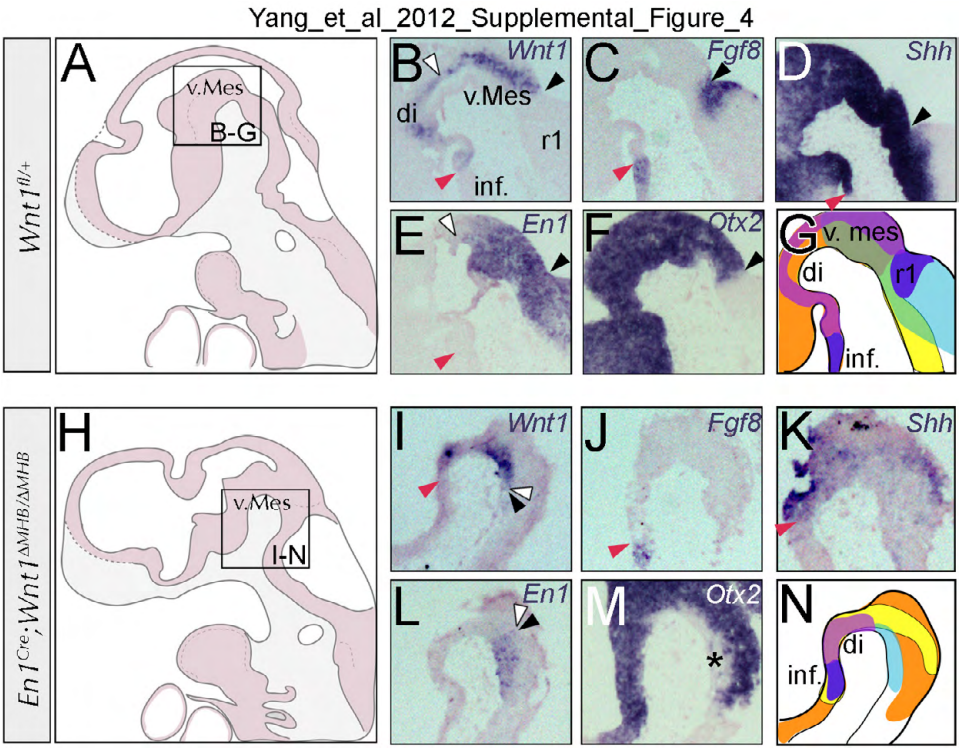

Fig. S4. Conditional deletion of *Wnt1* disrupted the molecular identity of the v.Mes. (**A-G**)The v.Mes and v.r1 of E10.5 control embryos were analyzed by *in situ* hybridization. (A) Regions of analysis. (B) *Wnt1* was in the v.Mes (between white and black arrowheads) and the di (between red and white 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**) $En1^{Cre}$;$Wnt1^{\Delta MHB/\Delta MHB}$ 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 $En1^{Cre}$ 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).

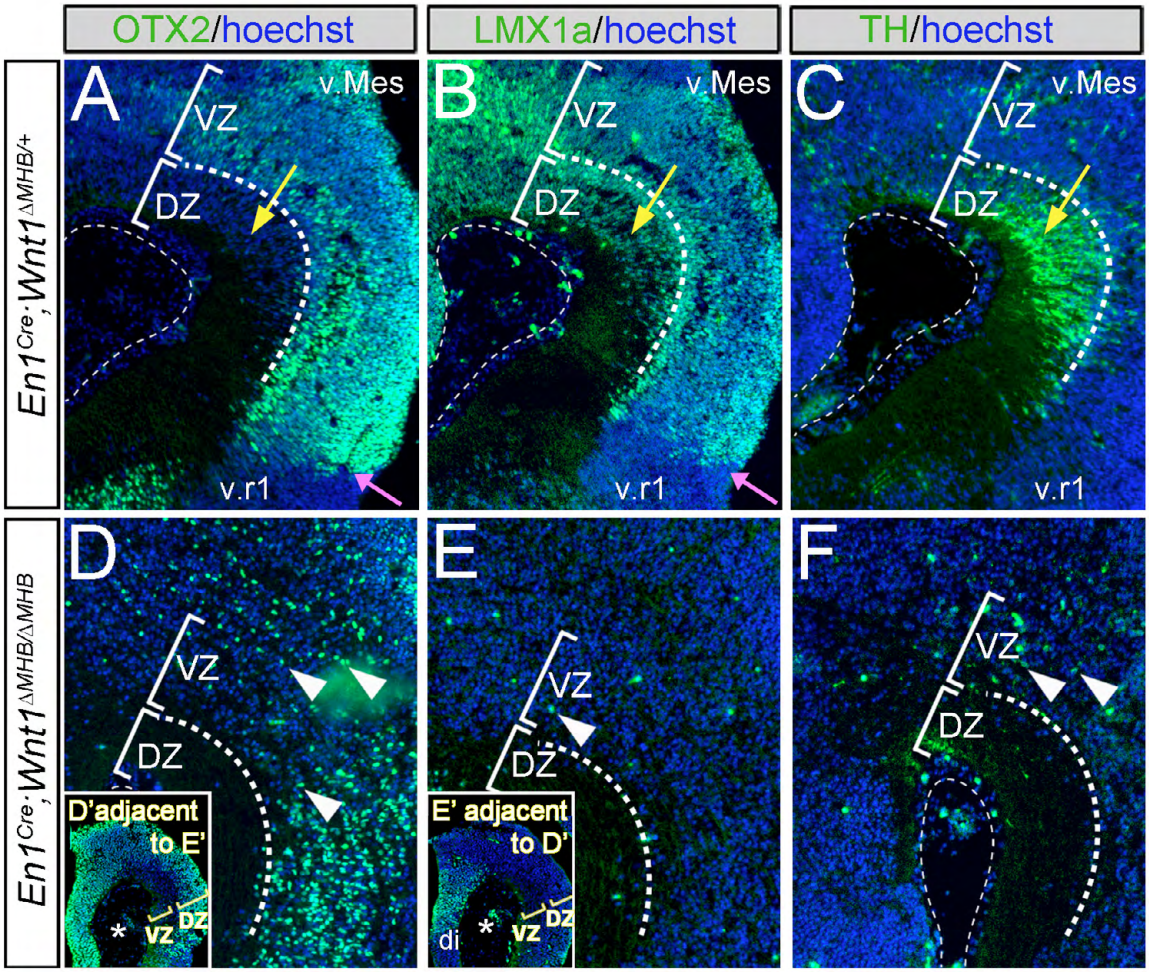

Fig. S5. Comparison of markers in embryos with early deletion of *Wnt1*. (**A-F**) Sagittal sections of control *En1*^{Cre};*Wnt1*$^{\Delta MHB/+}$ (A-C) and mutant *En1*^{Cre};*Wnt1*$^{\Delta MHB/\Delta MHB}$ (D-F) embryos at E12.5 were immunolabeled for OTX2, LMX1a or TH (markers are in green) and counterstained with Hoechst (blue) as indicated. (A,B) Controls had OTX2^{+} cells localized to the ventricular zone (VZ) and LMX1a^{+} cells in both the VZ and differentiating zone (DZ). (D,E) Mutants had OTX2^{+} cells scattered in the VZ. LMX1a was not detected in the v.Mes. (C) TH^{+} dopamine neurons were distributed in the DZ of controls. (D) Only a few TH^{+} 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*^{Cre};*Wnt1*$^{\Delta MHB/\Delta MHB}$ mutant sections shown in panels D and E (described above). OTX2 (D'), but not LMX1a (E') was detected in the v.Mes. LMX1a^{+} cells were observed in the diencephalon (di). TH^{+} 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.

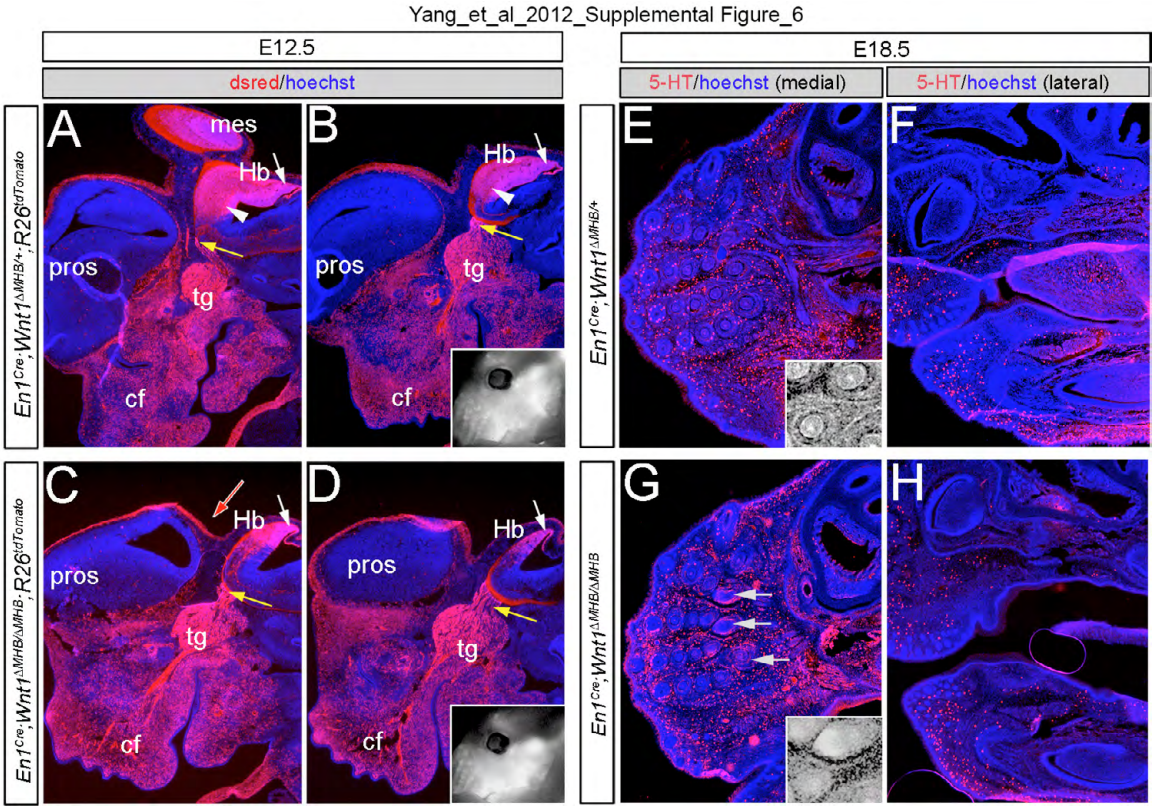

Fig. S6. Neural crest-derived structures assessed after conditional gene deletion and GIFM. (**A**,**B**) *En1*^{Cre};*Wnt1*$^{\Delta MHB/+}$;*R26*^{tdTomato} control embryo at E12.5 showing neural tube and neural crest structures derived from the *En1*-lineage (dsred^{+}, red) in lateral (A) and off-midline (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*$^{\Delta MHB/+\Delta MHB}$;*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.

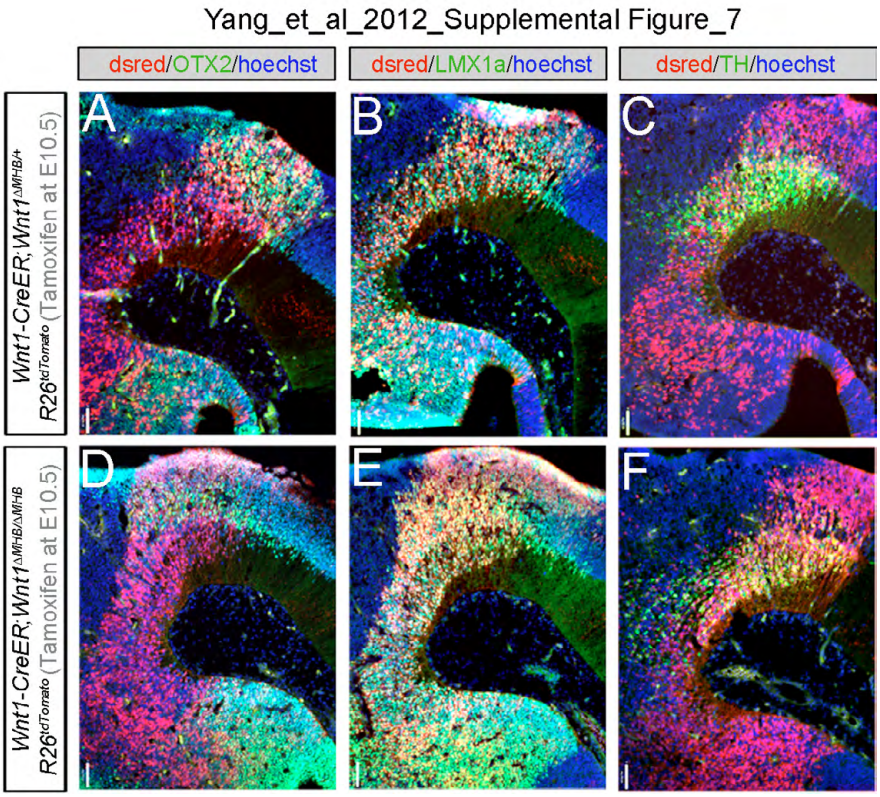

Fig. S7. *Wnt1* is temporally required for MbDA neuron development. Tamoxifen was given at E10.5 and embryos were analyzed at E12.5. (**A-C**) *Wnt1-CreER;Wnt1*$^{\Delta MHB/+}$*;R26*^{tdTomato} controls. Sections immunolabeled with antibodies recognizing indicated markers (green) and the lineage tracer (dsred^{+}, red) showed *Wnt1*-derived cells distributed in the v.Mes. (A,B) *Wnt1* lineage cells expressed OTX2 and LMX1a. (C) *Wnt1*-lineage derived TH^{+} MbDA neurons in the dz. The caudal limit of the MbDA neurons aligned with posterior *Wnt1* lineage-derived cells. (**D-F**) *Wnt1-CreER;Wnt1*$^{\Delta MHB/\Delta MHB}$*;R26*^{tdTomato} 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.

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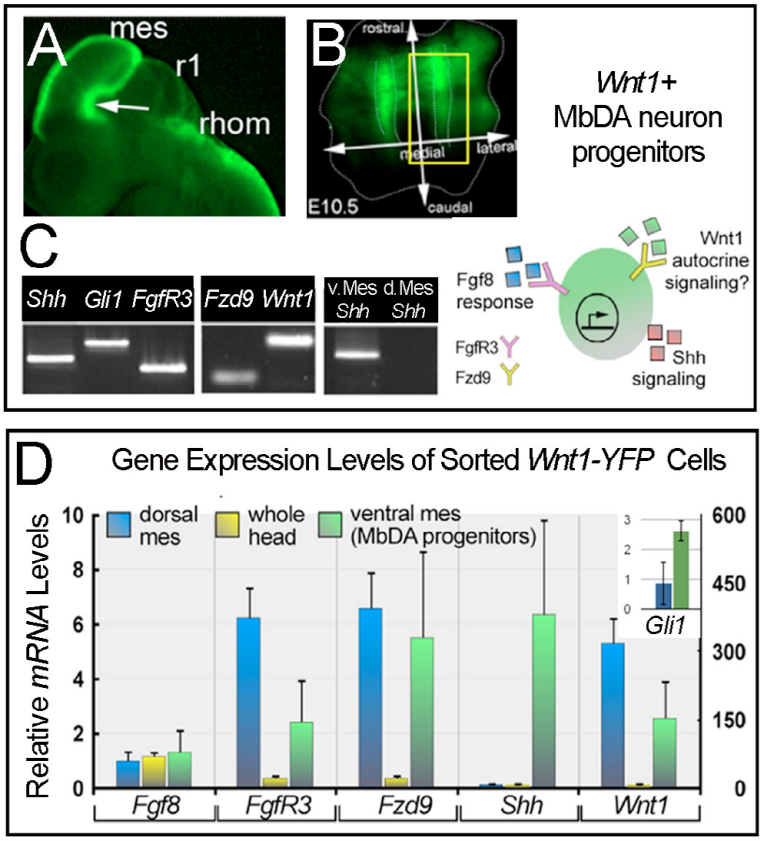

Fig. S8. Molecular characterization of the *Wnt1* lineage during development. (**A**) *Wnt1-Venus* embryo at E10.5 showing the location of putative MbDA neuron progenitors in the v.Mes (arrow). (**B**) The v.Mes was microdissected (Brown et al., 2009) to clearly identify the bilateral v.Mes YFP$^+$ progenitors, 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$^+$ 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$^+$ 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.

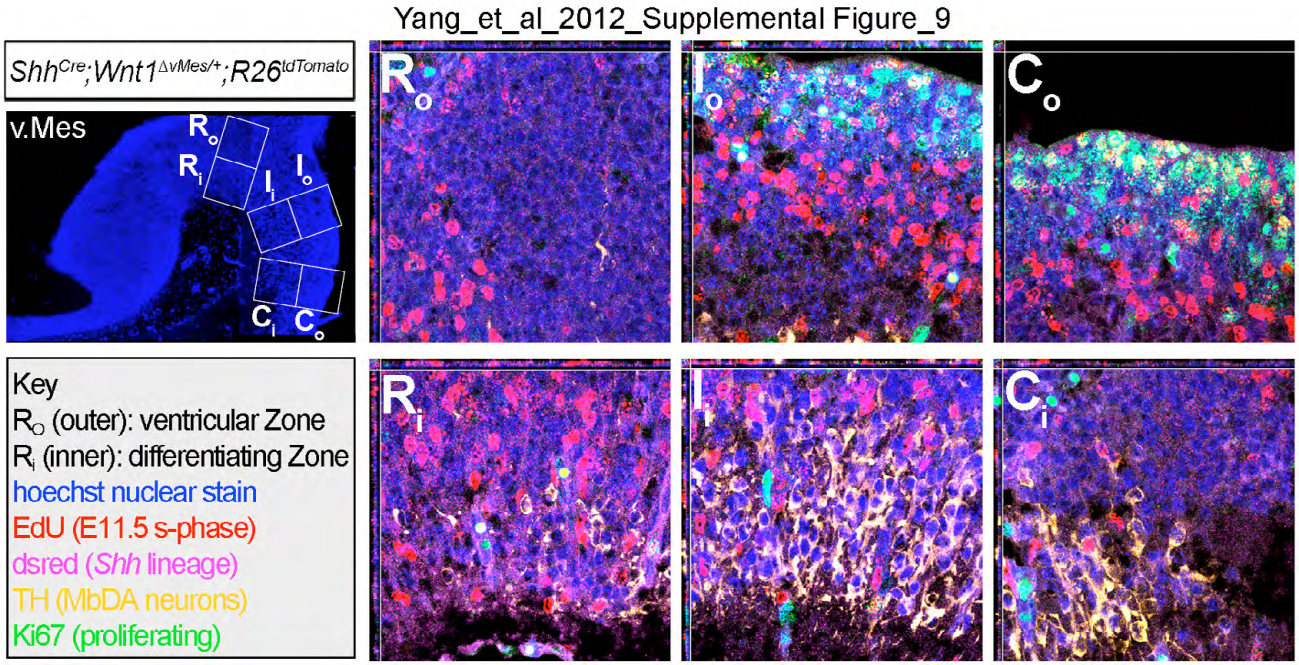

Fig. S9. Sampling scheme for quantitative cell cycle analysis. Representative image of the v.Mes. The boxes indicate where 40× *z*-series stacks were collected to sub-sample the tissue. The counting frames were organized in the following manner to analyze specific domains in the v.Mes along the rostral-to-caudal axis. Six counting frames were used and are designated as follows: rostral-outer (R_O), rostral-inner (R_i), intermediate-outer (I_O), intermediate-inner (I_i), caudal-outer (C_O) and caudal-inner (C_i). 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 c*assette (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	
Subcloning	AGATGTGCTAGTCATGAAGATGAACCGGGCTTGTTTCTCTGGCAACTAGG
	TGTCCTCCGGCTCTTCATTTACCTAGTTCTTGGTCAAAAGAGATGTCTAG
LoxP	CTGATGACAGAACAGAAGAGATTAGCAGGCTATCAACACGTGGGATGTAT
	CGCCAGCAAGTCACTTTTATCTTTCAAAAGTGTGCCATGGAGCCATCTCA
LoxP-FRT	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 *loxP*
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 *Neo*
5	5′-ATG GTT TAG TTC CTC ACC TTG TCG-3′	
6	5′-ACA GCA ACC ACA GTC GTC-3′	Upstream *loxP* once *Neo* is deleted
7	5′-TTT GGT TCA AGA CCT AAG GGA CAC-3′	
8	5′-GAA GCC CGT ACT TCA CAA CC-3′	Validates *Amp*
9	5′-CGA CAC GGA AAT GTT GAA TAC-3′	

Table S3. Sequence of primers used for qRT-PCR		
Fgf8	Fwd: 5′-CATTGTGGAGACCGATACTTTTGG-3′	122 bp amplicon
	Rev: 5′-AGTCCTTGCCTTTGCCGTTG-3′	
FgfR3	Fwd: 5′-ATCCTCGGGAGATGACGAAGAC-3′	314 bp amplicon
	Rev: 5′-GGATGCTGCCAAACTTGTTCTC-3′	
Fzd9	Fwd: 5′-CATCATGGAGCAATTCAATTTCG-3′	51 bp amplicon
	Rev: 5′-CGGGCACAGTCGAGTGAGT-3′	
Wnt1	Fwd: 5′-GGCCGAGATTCGTGGACT-3′	
	Rev: 5′-GCATCTCAGAGAACACGGTCG-3′	
Shh	Fwd: 5′- GGAAGATCACAAGAAACTCCGAAC-3′	354 bp amplicon
	Rev: 5′- GGATGCGAGCTTTGGATTCATAG-3′	
Gli1	Fwd: 5′- TGTTGTGGGAGGGAAGAAAC -3′	102 bp amplicon
	Rev: 5′- TGGCAGGGCTCTGACTAACT -3′	