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Inactivation of the β-catenin gene by *Wnt1-Cre*-mediated deletion results in dramatic brain malformation and failure of craniofacial development

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SUMMARY

β-Catenin is a central component of both the cadherin-catenin cell adhesion complex and the Wnt signaling pathway. We have investigated the role of β-catenin during brain morphogenesis, by specifically inactivating the β-catenin gene in the region of *Wnt1* expression. To achieve this, mice with a conditional ('floxed') allele of β-catenin with required exons flanked by *loxP* recombination sequences were intercrossed with transgenic mice that expressed Cre recombinase under control of *Wnt1* regulatory sequences. β-catenin gene deletion resulted in dramatic brain malformation and failure of craniofacial development. Absence of part of the midbrain and all of the cerebellum is reminiscent of the conventional *Wnt1* knockout (*Wnt1*$^{-/-}$), suggesting that Wnt1 acts through β-catenin in controlling midbrain-hindbrain development. The craniofacial phenotype, not observed in embryos that lack Wnt1, indicates a role for β-catenin in the fate of neural crest cells. Analysis of neural tube explants shows that β-catenin is efficiently deleted in migrating neural crest cell precursors. This, together with an increased apoptosis in cells migrating to the cranial ganglia and in areas of prechondrogenic condensations, suggests that removal of β-catenin affects neural crest cell survival and/or differentiation. Our results demonstrate the pivotal role of β-catenin in morphogenetic processes during brain and craniofacial development.

Key words: β-catenin, Cell adhesion, *Wnt1*, Conditional inactivation, CNS development, Neural crest, Mouse

INTRODUCTION

β-catenin was originally identified complexed with the cell adhesion molecule (CAM) E-cadherin (Vestweber and Kemler, 1984; Ozawa et al., 1989; Nagafuchi and Takeichi, 1989). Subsequently, β-catenin was found to bind directly to the cytoplasmic domain of E-cadherin and to α-catenin, linking this adhesion complex to the actin cytoskeleton (Aberle et al., 1994; Aberle et al., 1996a; Hülsken et al., 1994; Jou et al., 1995; Rimm et al., 1995). Compelling evidence has since been provided that the E-cadherin/catenin complex is crucial for epithelial cell polarity and function (Aberle et al., 1996b). Furthermore, mutations in components of the E-cadherin/catenin complex are correlated with increased invasiveness and metastasis of tumor cells (Berx et al., 1998).

The homology of β-catenin with *Drosophila* Armadillo (Arm) suggested the now well-established fact that β-catenin – like Arm – is part of the Wingless/Wnt (Wg/Wnt) signaling pathway (McCrea et al., 1991; Butz et al., 1992). Wnts act as signaling molecules and are implicated in many developmental processes, including cell fate specification, polarity, migration and proliferation (Gonzalez et al., 1991; Jue et al., 1992; Cadigan and Nusse, 1997). Upon binding to cell surface receptors, Wnts initiate an intracellular cascade that, via several intermediate steps, leads to the translocation of β-catenin to the nucleus. There, together with transcription factors of the T-cell factor/lymphoid enhancer-binding factor 1 (TCF/LEF1) family, β-catenin regulates expression of target genes (reviewed by Eastman and Grosschedl, 1999; Miller et al., 1999).

Many vertebrate Wnts are expressed in the embryonic central nervous system (CNS) (Parr et al., 1993; Hollyday et al., 1995). In the mouse, beginning 8.5 days post coitum (dpc), *Wnt1* and *Wnt3a* are expressed along the dorsal midline of the neural tube, suggesting a role in regional specification of the neural tube (Roelink and Nusse, 1991; Parr et al., 1993). By 9.5 dpc at least seven Wnts are expressed in the presumptive brain and spinal cord, including *Wnt1*, *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt5a*, *Wnt7a* and *Wnt7b* (Parr et al., 1993; Salinas and Nusse, 1992), suggesting multiple and complex patterns of Wnt signaling.

Wnt1 plays an important role in the anterior-posterior

patterning of the CNS (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Inactivation of *Wnt1* results in failure of midbrain and rostral hindbrain development (McMahon et al., 1992; Mastick et al., 1996; Serbedzija et al., 1996). Wnt1 acts by maintaining the expression of the transcription factor engrailed 1 (En1) in the caudal midbrain and rostral hindbrain (Danielian and McMahon, 1996). Absence of phenotype within the spinal cord of the *Wnt1*$^{-/-}$ mutant and the lack of neural tube phenotype for knockouts of several Wnt genes known to be expressed in the embryonic neural tube suggest redundancy between Wnt signals. Combined deletion of *Wnt1* and *Wnt3a* (*Wnt1-3a* double mutant) has revealed additional roles for Wnt signaling in both the brain and spinal cord and in neural crest derivatives (Ikeya et al., 1997; S. Lee, M. I. and A. P. M., unpublished).

Lack of β-catenin affects mouse development at gastrulation with failure of both mesoderm development and axis formation (Haegel et al., 1995; Hülsken et al., 2000). This early embryonic lethality has precluded studies of β-catenin during later development and organogenesis. To determine the role of β-catenin as a mediator of Wnt signaling during brain development, we have conditionally inactivated the β-catenin gene (*Catnb* – Mouse genome Informatics) using the Cre/*loxP* recombination system of bacteriophage P1 (Gu et al., 1994).

We show that the specific inactivation of the β-catenin gene in the domain of *Wnt1* expression results in dramatic brain malformation and failure of craniofacial development.

MATERIALS AND METHODS

Introduction of *loxP* sites into the β-catenin gene and generation of *β-catenin*$^{flox/+}$ and *β-catenin*$^{floxdel/+}$ mice

To construct the targeting vector, a *Sal*I/*Xho*I fragment from pBS112Sxneo/tk (Achatz et al., 1997) was cloned into the unique *Nsi*I site of the previously cloned mouse β-catenin genomic sequence pMBC.G1-E7 (originating from a 129/Sv mouse strain) (Haegel et al., 1995). A third *loxP* site was introduced into a *Sph*I site using two oligonucleotides (RM15 5′ CCT GCA GAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATG CAT G 3′; RM16 5′ CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATC TGC AGG CAT G 3′). The targeting vector was linearized with *Not*I and electroporated into R1 ES cells (Nagy et al., 1993). G418-resistant clones were screened for homologous recombinants by a nested PCR using primers RM34 (5′ TGG TTC GTG GGG GTT ATT ATT TTG 3′) and RM35 (5′ CAT TTT CCG CTT CTA CTT GGT TCT 3′) (1.64 kb PCR product) in the initial reaction and then RM37 (5′ GCA GGT CGT CGA GAT CCG GAA CC 3′) and RM38 (5′ TCA CTG GGG AGA ACA CCT TAA C 3′) (1.48 kb PCR product) (Fig. 1A). Correct homologous recombination was confirmed by Southern analysis of isolated genomic DNAs digested with *Nsi*I or *Eco*RI and probed with probes B (Fig. 1A,C) and A (Fig. 1A,B), respectively. A single integration was confirmed by probing *Ssp*I digested genomic DNA with the neo/tk probe originating from pBS112Sxneo/tk. Positive clones with all three *loxP* sites were transiently transfected with the Cre-encoding plasmid pMCcreN (Achatz et al., 1997) and selected with gancyclovir. Colonies surviving the selection were genotyped by Southern blotting using probe pMBC.G1-E7 (Fig. 1A,D). Two *β-catenin*$^{flox/+}$ ES cell clones (2A1 and 3-24) were microinjected into C57/BL6 host blastocysts and chimeras obtained were bred with C57/BL6 mice. The floxdel allele was created by mating heterozygous floxed mice with the *CMV-Cre* deleter mice (Schwenk et al., 1995). The *Wnt1-Cre* transgenic line has been described previously (Danielian et al., 1998).

DNA preparation and analysis

For the identification of the β-catenin alleles and the *Wnt1-Cre* transgene, DNA was isolated from yolk sac of embryos and tail biopsies of adults. After lysis in buffer containing proteinase K, genomic DNA was precipitated using isopropanol and dissolved in TE buffer; 0.5 μg of genomic DNA was used for PCR. The 5′ and 3′ primers used for detecting the *Cre* gene, pCre1 (5′ ATG CCC AAG AAG AAG AGG AAG GT 3′) and antisense primer pCre2as (5′ GAA ATC AGT GCG TTC GAA CGC TAG A 3′), generate a 447 bp product. To detect the β-catenin floxed allele, sense primer RM41 (5′ AAG GTA GAG TGA TGA AAG TTG TT 3′) and antisense primer RM42 (5′ CAC CAT GTC CTC TGT CTA TTC 3′) were used, generating 324 bp and 221 bp products from the floxed and wild-type alleles, respectively. The floxdel allele was screened using sense primer RM68 (5′ AAT CAC AGG GAC TTC CAT ACC AG 3′) and antisense primer RM69 (5′ GCC CAG CCT TAG CCC AAC T 3′), which generate a 631 bp product from the floxdel allele. Different combinations of floxed and/or floxdel β-catenin alleles were identified by PCR using primers RM41, RM42 and RM43 (5′ TAC ACT ATT GAA TCA CAG GGA CTT 3′), resulting in products of 221 bp for the wild-type allele, 324 bp for the floxed allele and 500 bp for the floxdel allele. *Wnt1*$^{-/-}$ and *Wnt1*$^{-/-}$*; Wnt3a*$^{-/-}$ mutants were genotyped as described (McMahon and Bradley, 1990; Takada et al., 1994).

Mating scheme and embryological techniques

In the mating scheme devised, only one floxed allele needs to undergo recombination to create tissue null for the gene. In a first cross *Wnt1-Cre* transgenic mice were mated with mice heterozygous for the β-catenin floxdel allele. The offspring inheriting both a *Wnt1-Cre* and a floxdel allele were then mated with homozygous floxed β-catenin mice to obtain embryos with the *Wnt1-Cre* transgene together with one floxed and one floxdel allele.

For histological examination, embryos were collected in PBS, fixed in Bouin's fixative, dehydrated, embedded in paraffin and sectioned at 2 μm. Sections were dewaxed, rehydrated and stained with Hematoxylin and Eosin. Whole-mount in situ hybridization was performed (Parr et al., 1993; as modified by Knecht et al., 1995), using digoxigenin-labeled probes for *Ap2* (Mitchell et al., 1991), the gene for cadherin 6 (Inoue et al., 1997), *Crabp1* (Stoner and Gudas, 1989), *Cre* (Achatz et al., 1997), *En1* (Wurst et al., 1994), *Fgf8* (Tanaka et al., 1992), *Hoxa2* (Mallo, 1997), *Isl1* (Neidhardt et al., 2000), *Otx2* (Simeone et al., 1992) and *Wnt1* (Parr et al., 1993),

Whole-mount immunohistochemistry was performed with 2H3 anti-neurofilament antibody (from the Developmental Hybridoma Bank at the NICHD) at 10.5 dpc, as described (Swiatek and Gridley, 1993).

Preparation of skeletons was as described previously (Mallo and Brändlin, 1997). Briefly, 18.5 dpc embryos were eviscerated, skinned, fixed in ethanol and stained with Alcian Blue and Alizarin Red.

Apoptosis was visualized using whole-mount TUNEL assays (Kanzler et al., 2000).

ES cell and neural crest cultures and immunocytochemistry

ES cells were isolated according to Nagy et al. (Nagy et al., 1993).

Neural crest cultures were performed from 9.25 dpc embryos as described (Sommer et al., 1995), using standard medium conditions (Hagedorn et al., 1999).

Double immunofluorescence tests were carried out with rabbit anti-p75 (1:300 dilution; Chemicon) and Cy3-conjugated goat anti-rabbit IgG (1:500 dilution; Jackson ImmunoResearch Laboratories), and with mouse monoclonal anti-β-catenin antibody (1:500 dilution; Transduction Laboratories) and FITC-conjugated anti-mouse IgG antibody (1:200 dilution; Vector Laboratories) as described (Sommer et al., 1995).

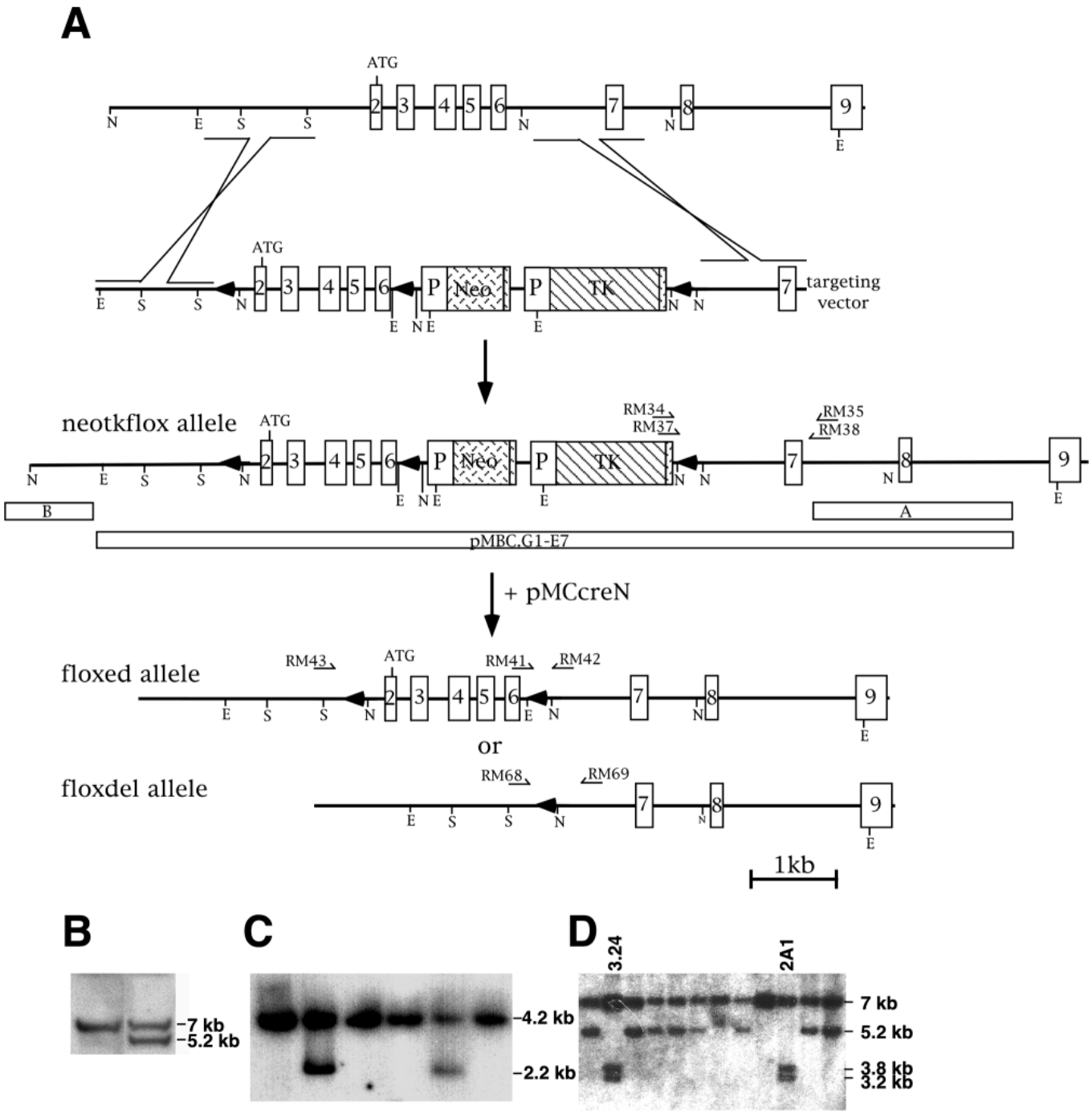

Fig. 1. Targeting strategy for conditional inactivation of the gene for β-catenin. (A) Restriction map of the mouse β-catenin locus, the targeting construct and the homologous recombined allele (neotkflox). The floxed and floxdel alleles were generated by transient transfection of the *CMV-Cre* expression plasmid. Exons are white boxes (2 to 9) and intronic sequences are solid lines. The neo and tk cassettes are patterned boxes. Black triangles indicate *loxP* sequences. The positions of probes (A, B and pMBC.G1-E7), restriction enzyme sites (E, *Eco*RI; N, *Nsi*I; S, *Ssp*I) and primers used for PCR analysis are shown. (B-D) Southern blotting for homologous recombination in R1 ES cells. (B) DNAs were isolated from ES cells, digested with *Eco*RI and probed with probe A to screen for correct 3′ recombination. The 5.2 kb and 7 kb bands represent the targeted and wild-type alleles, respectively. (C) For correct 5′ recombination, the DNA was digested with *Nsi*I and probed with probe B. Correct recombinants generated a 2.2 kb band in addition to the 4.2 kb band from the wild-type allele. (D) Cre-mediated deletion in ES cells. DNAs were isolated from R1 ES cells that had been transiently transfected with the *CMV-Cre* plasmid. DNA digest with *Eco*RI and Southern blotting with probe pMBC.G1-E7 confirmed the generation of floxed and floxdel alleles. ES cell clones heterozygous for the floxed allele generated a 7 kb (wild-type) band and two bands (3.8 and 3.2 kb) for the floxed allele. The floxdel allele generated a 5.2 kb band. Clones 2A1 and 3.24 were used to generate germline chimeras.

RESULTS

Generation of floxed and floxdel β-catenin mice

To generate a floxed β-catenin allele, a targeting vector was designed such that exons 2 (which contains the ATG translational start) to 6 of the β-catenin gene were flanked by two *loxP* sites (Fig. 1A). This vector was electroporated into R1 embryonic stem (ES) cells (Nagy et al., 1993) and 3 out of 200 neo^r clones were isolated as homologous recombinants. Having undergone appropriate homologous recombination, as assayed by Southern analysis (see Materials and Methods and Fig. 1B,C), one of the clones was transiently transfected with the *Cre*-encoding plasmid pMCcreN in order to excise the neo/tk selection markers. About 20% of surviving ES cell clones had deleted the selection markers but retained exons 2 to 6 (see floxed allele, Fig. 1A and Southern analysis, Fig. 1D). Two independent floxed β-catenin ES cell clones were used to generate germline chimeras, and heterozygous floxed mice originating from both clones were bred to homozygosity. Homozygous animals from both clones were viable, fertile and showed no noticeable phenotype.

By mating floxed β-catenin mice with *CMV-Cre* deleter mice (Schwenk et al., 1995), mice heterozygous for the recombined floxed β-catenin allele (floxdel allele) were generated. Embryos homozygous for the β-catenin floxdel allele died at gastrulation with a phenotype similar to that previously reported for β-catenin null embryos (Haegel et al., 1995; Hülsken et al., 2000; data not shown). In blastocyst cultures, the inner cell mass (ICM) of β-catenin floxdel homozygous embryos exhibited a clear cell adhesion defect compared to wild-type embryos (Fig. 2B). Thus, the Cre-mediated recombination was able to convert the β-catenin floxed allele into a floxdel allele unable to generate a functional β-catenin protein (see also Fig. 7C-F).

Cre expression in 9-11.5 dpc embryos obtained from *Wnt1-Cre* transgenic mice was tested by whole-mount in situ hybridization analysis and confirmed earlier reports including expression of *Cre* in neural crest cell (NCC) precursors (Danielian et al., 1992; Echelard et al., 1994; Chai et al., 2000; not shown). The ability of Cre to recombine the β-catenin floxed allele was tested by mating *Wnt1-Cre* hemizygous mice with *β-catenin*$^{floxed/floxed}$ mice. The generation of the floxdel allele was examined by PCR analysis of embryonic DNA. The floxdel allele was detected as early as 8.5 dpc and only in embryos positive for *Wnt1-Cre*, indicating that the Cre is capable of recombining the *loxP* sites within the β-catenin gene (Fig. 2C).

Failure of midbrain-hindbrain development in β-catenin mutant embryos

Compound heterozygotes for the β-catenin floxed and floxdel alleles and carrying the *Wnt1-Cre* transgene were generated (β-catenin mutant embryos), while littermates (which inherited the incomplete combination of the above alleles) served as wild-type controls.

At 18.5 dpc, all of the β-catenin mutant fetuses exhibited dramatic brain malformation and no craniofacial development

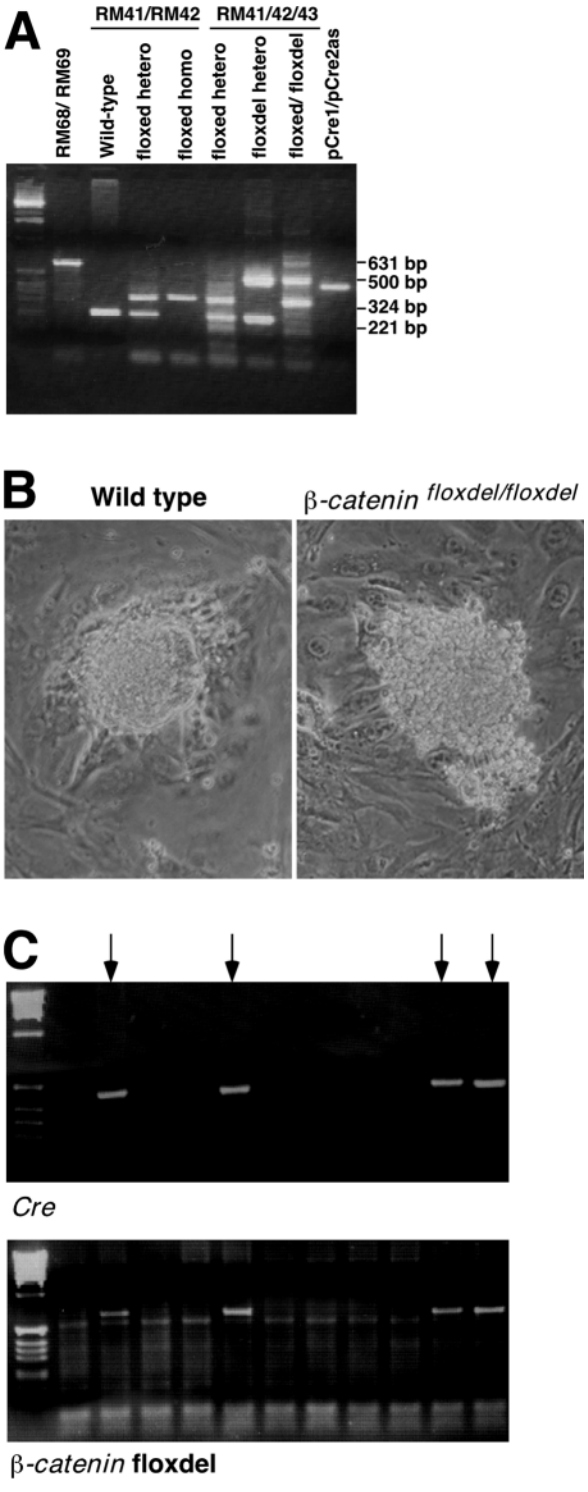

Fig. 2. Genotyping of mice and embryos, adhesive defects in *β-catenin*$^{floxdel/floxdel}$ embryos and analysis of the *Wnt1-Cre* transgene. (A) Identification of the different β-catenin alleles and the *Cre* transgene in vivo by PCR analysis. PCR amplification using primers RM68/RM69 generates a 631 bp product for the β-catenin floxdel allele. Primers RM41/RM42 amplify both the β-catenin floxed allele (324 bp) and the wild-type allele (221 bp). A combination of primers RM41/RM42/RM43 results in 221 bp (wild-type), 324 bp (floxed) and 500 bp (floxdel) products. (B) Cultured *β-catenin*$^{floxdel/floxdel}$ embryos exhibited a poorly adherent ICM. (C) The Cre enzyme is capable of recombining the β-catenin floxed allele in vivo. PCR confirming the presence of the recombined β-catenin allele (floxdel allele, bottom PCR using primers RM68/RM69) in 8.5 dpc embryos from *Wnt1-Cre*/+ × *β-catenin*$^{flox/flox}$ crosses which also inherited the *Cre* transgene (top PCR).

(Fig. 3A,B). Mutant embryos and fetuses were recovered in 25% of total embryos, but no mutant newborns were found, indicating that β-catenin mutants die around birth. The mutant phenotype could be recognized at 9.5 dpc (Fig. 3C,D) by a shortened neural tube. At 10.5 dpc, the mutant CNS was shorter along the antero-posterior axis, suggesting that parts of the midbrain and/or the anterior hindbrain were missing or reduced in size, and the isthmic border between the midbrain and rhombomere 1 (r1) was not visible (Fig. 3E,F). The extent of this deletion varied slightly among mutant embryos. In addition, the telencephalon appeared somewhat larger than in the wild type, while the walls of the cephalic vesicles looked thinner. There was often abnormal accumulation of blood within the cranial region. From 10.5 to 18.5 dpc, brain morphogenesis was grossly abnormal and craniofacial structures did not develop at all in β-catenin mutant embryos (data not shown). The mutant phenotype was consistent at each developmental stage with only slight variations in the extent of brain malformations at earlier stages.

In histological sections at 12.5 dpc of wild-type embryos, the telencephalic vesicles, diencephalon and midbrain are visible (Fig. 3G), the cerebellar primordium has formed from the dorsal metencephalon, and the choroid plexus marks the metencephalic-myelencephalic junction. In contrast, 12.5 dpc β-catenin mutant embryos had no discernible midbrain and neither a cerebellum nor a choroid plexus (Fig. 3H). This is reminiscent of the *Wnt1*$^{-/-}$ phenotype (McMahon and Bradley, 1990), providing additional support for the idea that Wnt1 acts through β-catenin. Remarkably, the β-catenin mutant phenotype appeared to be more extended than the *Wnt1*$^{-/-}$ phenotype; the forebrain did not develop properly and craniofacial structures were absent, suggesting an additional role for β-catenin in NCC migration and/or differentiation.

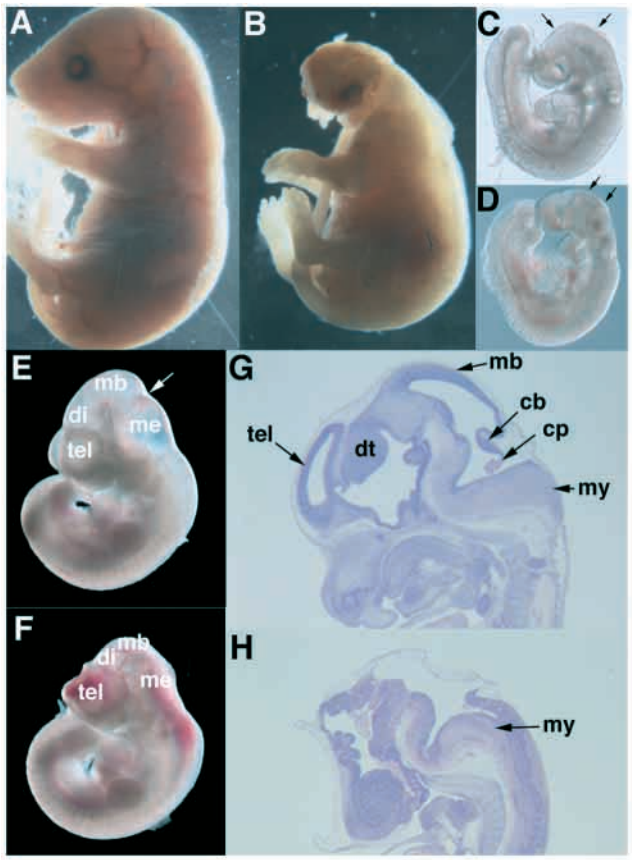

Fig. 3. CNS defects and absence of craniofacial development in β-catenin mutant embryos. Wild-type (A,C,E,G) and β-catenin mutant (B,D,F,H) embryos at 18.5 dpc (A,B), 9.5 dpc (C,D), 10.5 dpc (E,F), and Hematoxylin and Eosin-stained sagittal sections at 12.5 dpc (G,H). Small arrows in C,D indicate the deleted region in the β-catenin mutant. Arrow in E indicates the isthmus. cb, cerebellum; cp, choroid plexus; di, diencephalon; dt, dorsal thalamus; mb, midbrain; me, metencephalon; my, myelencephalon; tel, telencephalon.

Analysis of early midbrain and hindbrain markers

Expression analysis of early midbrain and hindbrain markers has shown that the *Wnt1*$^{-/-}$ phenotype results from the early deletion of the midbrain and a subsequent loss of rostral hindbrain (McMahon et al., 1992). A similar analysis was undertaken with β-catenin mutant embryos by whole-mount in situ hybridization at 9.5 dpc (Fig. 4). At this stage, *Otx2* expression in wild-type embryos was detected throughout the forebrain and midbrain with a sharp boundary at the mesencephalic-metencephalic junction (Fig. 4A, arrow) (Simeone et al., 1993; Millet et al., 1996). In β-catenin mutant embryos, *Otx2* expression was reduced to the anterior forebrain, supporting the notion that part of the midbrain was missing (Fig. 4B). *Wnt1* is normally expressed in a transverse band at the posterior end of the midbrain and in a stripe along the dorsal midline of the mesencephalon, diencephalon and hindbrain posterior to the cerebellar anlage, with a characteristic gap of expression in the r1 region of the metencephalon (Wilkinson et al., 1987; McMahon et al., 1992; Parr et al., 1993; Fig. 4C). In β-catenin mutant embryos, Wnt1 expression was continuous between the remaining midbrain and caudal hindbrain, suggesting that the r1 region had been lost (Fig. 4D). The transverse band of expression at the dorsal midbrain was reduced to a small dorsal patch, whereas the stripe along the dorsal midline was widened. At 9.5 dpc, *Fgf8* is normally expressed in a sharp transverse stripe at the anterior boundary of the hindbrain immediately posterior to the *Wnt1*-expressing cells at the mesencephalic-metencephalic junction, and is also found in the commissural plate of the telencephalon, the dorsal region of the midbrain-forebrain boundary, the limb buds and the somites (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; Fig. 4E). In β-catenin mutants, the stripe of *Fgf8* expression in the anterior hindbrain was reduced to a small dorsal spot (Fig. 4F). The pattern of *Fgf8* expression, however, appeared normal in the forebrain and other regions of mutant embryos. Between 9 and 10 dpc, *En1* expression normally covers the isthmus, together with a large portion of the midbrain and the metencephalon up to r1 and r2 (Davidson et al., 1988; Davis and Joyner, 1988; Davis et al., 1991) (Fig. 4G). In the β-catenin mutant, *En1*-expressing cells were still present at 9 dpc (data not shown), but *En1* expression was generally lost by 9.5 dpc (Fig. 4H). Thus by 9.5 dpc the region of the caudal midbrain and anterior hindbrain was significantly affected in β-catenin mutant embryos.

Lack of skeletal structures derived from cranial neural crest

To analyze bone formation in the head, skeletal preparations

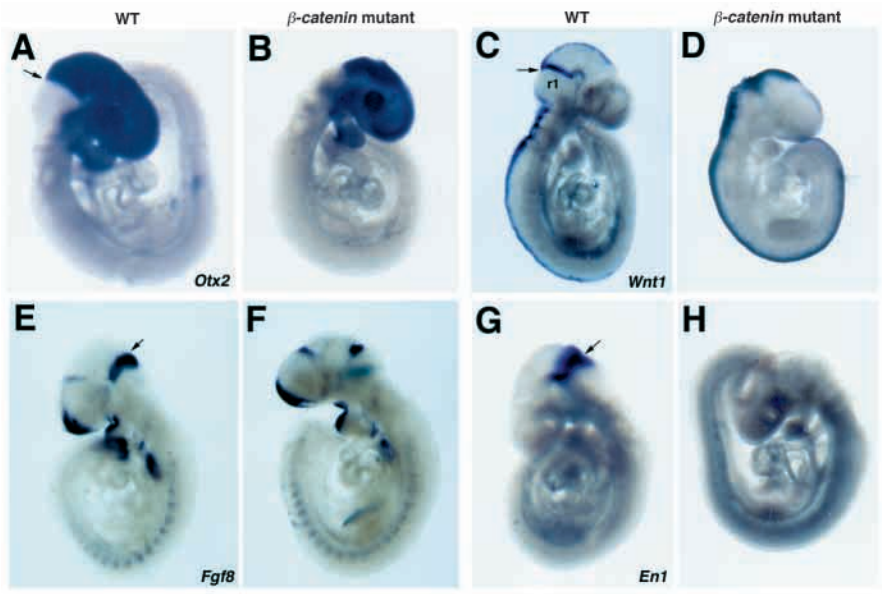

Fig. 4. Whole-mount in situ hybridization with early midbrain-hindbrain junction markers in 9.5 dpc wild-type (A,C,E,G) versus β-catenin mutant (B,D,F,H) embryos reveals that part of the mutant midbrain and rostral metencephalon are missing. *Otx2* (A,B); *Wnt1* (C,D); *Fgf8* (E,F); *En1* (G,H). Arrows point at the isthmic constriction. The reduced expression domain of *Otx2* in the β-catenin mutant (B) compared with the wild type (A) indicates that part of the midbrain is missing; the continuous expression of *Wnt1* in the dorsal neural tube (D) suggests that the anterior hindbrain region (r1 in C) is lost. Expression of *Fgf8* in the anterior hindbrain (E) is reduced to a small dorsal patch in the mutant (F). *En1* wild-type expression spans the isthmus (G) and much of the midbrain and the anterior metencephalon; it is completely lost by 9.5 dpc in the β-catenin mutant (H).

from 18.5 dpc wild-type and β-catenin mutant embryos were compared (Fig. 5). The trunk skeleton, including the vertebral column up to the atlas bone (arrowhead in Fig. 5A,B), was unaffected in the mutant embryos (data not shown), while in the head region most of the bones derived from cranial NCCs were absent (Fig. 5B,D). Bones that remained were those predominantly derived from the mesenchyme, e.g. the otic vesicle, basioccipital and exoccipital bones (compare Fig. 5C with Fig. 5D), but no supraoccipital bone was found (compare Fig. 5A with Fig. 5B). Bones and cartilages from dorsal midbrain and of r1 origin, i.e. the maxilla, mandible and tympanic ring (visible in Fig. 5A), were absent. The only remaining element from these regions was a vestigial Meckel's cartilage (r in Fig. 5B). Structures originating from r4 were either missing or affected; the styloid process and stapes (compare Fig. 5E with Fig. 5F) were absent, while the lesser horn of the hyoid bone of the laryngeal cartilages (compare Fig. 5G with Fig. 5H) was less affected. Structures originating from r6 and r7 in the hindbrain were less affected; the greater horn of the hyoid bone, the hyoid bone, and the thyroid and cricoid cartilages were present but malformed (Fig. 5H). In general, the formation of cranial NCC-derived skeletal structures is lacking or greatly perturbed in β-catenin mutant embryos.

Abnormalities in cranial and dorsal root ganglia in β-catenin mutant embryos

Wnt1 and Wnt3a are essential for the expansion of NCCs that give rise to the cranial ganglia and dorsal root ganglia (DRGs; Ikeya et al., 1997). In the conditional gene inactivation scheme undertaken here, β-catenin is eliminated in the dorsal midline of the CNS, where both *Wnt1* and *Wnt3a* are expressed and where the precursors of NCCs are generated (Chai et al., 2000; Jiang et al., 2000). We therefore analyzed the peripheral nervous system (PNS) at 10.5 dpc with an anti-neurofilament antibody by whole-mount immunostaining of wild-type (Fig. 6A,E), *Wnt1*$^{-/-}$ (Fig. 6B,F), *Wnt1-3a* double (Fig. 6C,G) and β-catenin mutant (Fig. 6D,H) embryos. In all three mutants (Fig. 6B-D), the oculomotor nerve (III in Fig. 6A) was missing and the tract of the mesencephalic nucleus of the trigeminal nerve (tmesV) was not distinctly formed, as already described in the *Wnt1*$^{-/-}$ mutant (Mastick et al., 1996). In both the *Wnt1-3a* double mutant and the β-catenin mutant, the connecting parts between the cranial ganglia and the hindbrain were poorly formed (Fig. 6C,D,G,H). In the β-catenin mutant, the trigeminal ganglion has lost its connections to the hindbrain. Instead, there was a mass around the exit point of this nerve (white arrow in Fig. 6D). The combined superior ganglion of nerves VII and VIII also formed an abnormal mass (black arrow in Fig. 6D). The roots of the glossopharyngeal (IX), vagus (X) and hypoglossal nerve (XII), were poorly formed, and the hypoglossal nerve was missing entirely. Thus, the cranial nerve and ganglion phenotypes were more severe in the β-catenin mutant than in the *Wnt1-3a* double mutant embryos. In the spinal cord, the first cervical DRG (arrowhead, Fig. 6G,H) was missing in both the β-catenin and the double mutant, while the more posterior DRGs were more severely affected in the double mutant than in the β-catenin mutant (asterisk in Fig. 6G,H). Whole-mount in situ hybridization for Cadherin6, a marker for glial NCC derivatives (Inoue et al., 1997), and *Isl1*, a marker for neuronal derivatives (Pfaff et al., 1996), stained NCC derivatives in the cranial ganglia and DRGs of β-catenin mutant embryos although staining was weaker compared with wild-type embryos (not shown).

NCC derivatives in branchial arches of β-catenin mutant embryos

Much of the head skeleton originates from cranial NCCs that delaminate from the dorsal neural tube, where *Wnt1* is expressed, and migrate along segmental pathways to the branchial arches, where they differentiate to give rise to craniofacial bones, cartilage and connective tissues (Le Douarin, 1982). To determine whether the defects in the β-catenin mutant could result from aberrant migration of NCCs into the arches, the expression of several NCC markers was examined by whole-mount in situ hybridization in 9-9.5 dpc embryos. AP2, a transcription factor essential for survival of

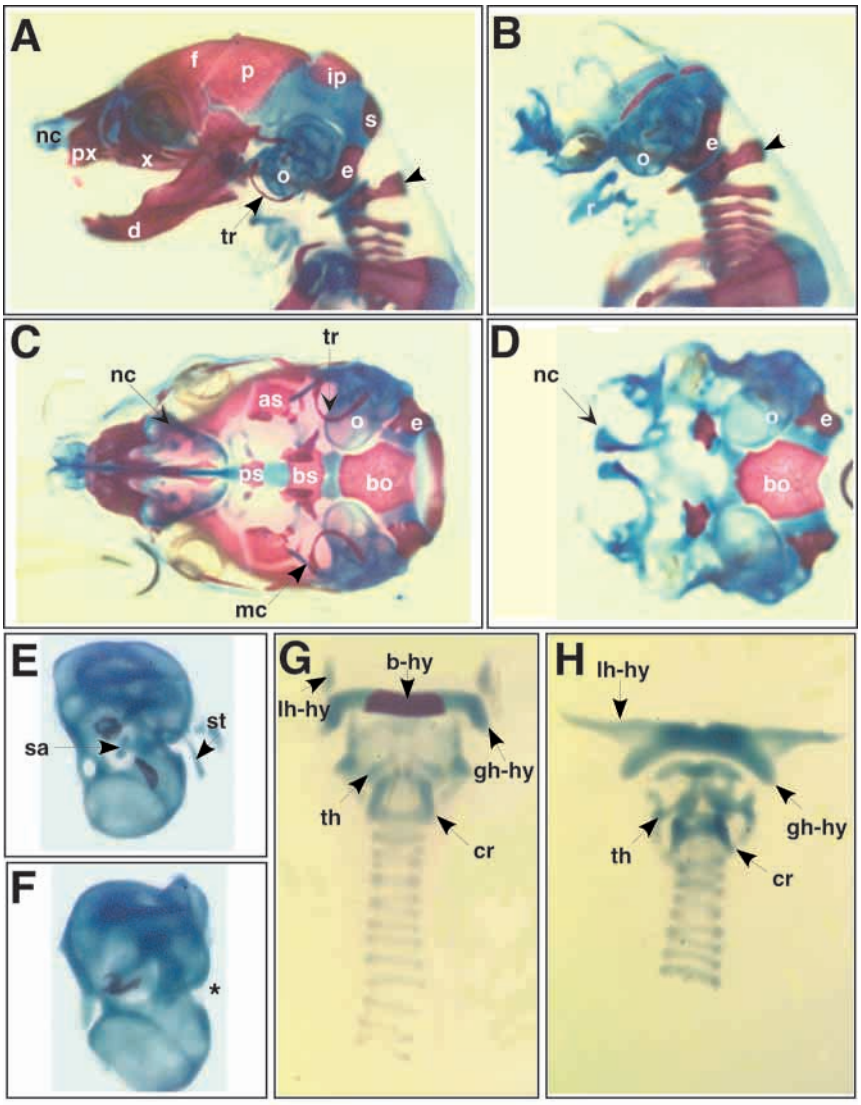

Fig. 5. Skeletal preparations of 18.5 dpc wild-type (A,C,E,G) and β-catenin mutant (B,D,F,H) fetuses stained with Alizarin Red and Alcian Blue; lateral (A,B) and basal (C,D) views. The mandible, maxilla and palatine bone were removed in wild type (C) to enhance the view of the cranial base. In the mutant most of the skeletal structures derived from the cranial NCCs are missing except the basioccipital (bo) and exoccipital (e) bones, and the otic capsule (o). The maxilla (x), mandible (d) and tympanic ring (t), derived from branchial arch 1, are completely lost. The only remaining element of arch 1 is vestigial Meckel's cartilage remnants (r). Dissection of the otic capsules and middle ear elements of wild-type (E) and β-catenin mutant (F) fetuses. The otic vesicle is present in the β-catenin mutant but not well formed, while the styloid process (asterisk in F) and middle ear elements are missing. Elements from the laryngeal skeleton are still present in the mutant (H versus wild-type G), but malformed. as, alisphenoid; b-hy, body of the hyoid bone; bs, basisphenoid; cr, cricoid cartilage; f, frontal bone; gh-hy, greater horn of the hyoid bone; i, incus; ip, interparietal bone; lh-hy, lesser horn of hyoid bone; m, malleus; mc, Meckel's cartilage; nc, nasal capsule; p, parietal bone; px, incisive (premaxillary) bone; s, supraoccipital bone; sa, stapes; st, styloid process; th, thyroid cartilage; tr, tympanic ring.

migratory NCCs (Mitchell et al., 1991; Schorle et al., 1996; Zhang et al., 1996), was found in streaks extending from r1 and r2 into arch 1 and r4 into arch 2 in both wild-type and β-catenin mutant embryos (Fig. 7A,B; I for arch 1 and II for arch 2). Comparable expression patterns in wild-type and β-catenin mutant embryos were also observed with other markers, i.e. *Crabp1* (Maden et al., 1992) and the most anteriorly expressed member of the Hox gene family, *Hoxa2* (Gendron-Maguire et al., 1993; Rijli et al., 1993; not shown), indicating that at least some NCC migration to the branchial arches occurs in β-catenin mutant embryos. To confirm that β-catenin is indeed deleted in NCCs of β-catenin mutant embryos, neural tube explant cultures were performed from 9.25 dpc wild-type and β-catenin mutant embryos. Outgrowing NCCs were stained for the NCC-specific marker p75 (Stemple and Anderson, 1992) and with a C-terminal-specific antibody for β-catenin in double-immunofluorescence experiments (Fig. 7C-F). The low-affinity neurotrophin receptor p75 stained equally well undifferentiated NCCs from wild-type and β-catenin mutant embryos. However, no staining with anti-β-catenin of p75-positive NCCs was observed in β-catenin mutant explants, demonstrating that *Wnt1-Cre* efficiently deleted the β-catenin gene and showing that these cells still exhibit migratory potential. Some non-NCCs, i.e. p75-negative cells, in β-catenin mutant cultures were positive for β-catenin, underlining the high degree of specificity of *Wnt1-Cre* deletion of the β-catenin gene in NCCs (not shown).

Increased apoptosis in β-catenin mutant embryos

The absence of craniofacial development could be due to an increase in apoptosis within the population of migratory NCCs. Therefore, TUNEL assays were performed at different stages of NCC migration. A first wave of NCC migration to the branchial arches occurs at 8.5 dpc (Serbedzija et al., 1990; Serbedzija et al., 1992). No apoptosis was observed in pathways of migratory NCCs and no increase of apoptotic cells was evident in the hindbrain of mutant embryos at this stage (Fig. 8A,B). At 9 dpc, increased apoptosis in β-catenin mutant embryos was evident in the hindbrain and in NCCs migrating to the cranial ganglia (bracket in Fig. 8D) as well as in the frontonasal mass (arrowhead in Fig. 8D), while the branchial arches had the same size as in wild-type embryos (arrow in Fig.

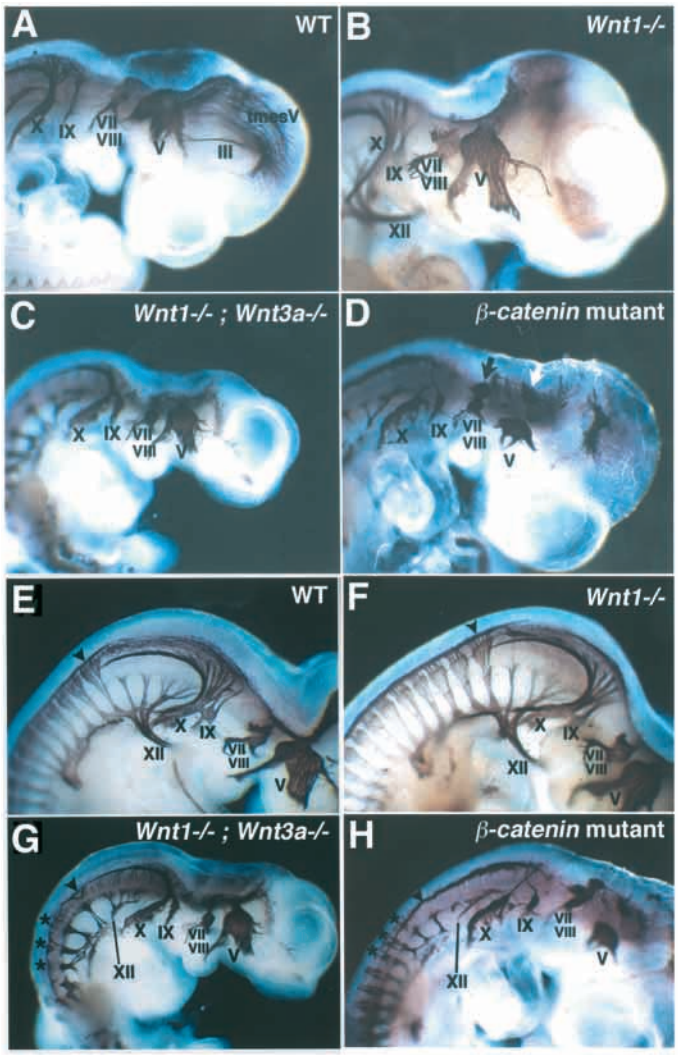

Fig. 6. Neurons in 10.5 dpc embryos were visualized by whole-mount immunostaining using anti-neurofilament antibody 2H3. Cranial region (A-D) and trunk region (E-H) of wild-type (A,E), *Wnt1*$^{-/-}$ (B,F), *Wnt1-3a* double mutant (C,G) and β-catenin mutant (D,H) embryos. In the three mutants (B,C,D), the oculomotor nerve (III) is absent as well as the tract of the mesencephalic nucleus of the trigeminal nerve (tmesV). While cranial ganglia are normal in *Wnt1*$^{-/-}$ embryos, the connecting parts between the cranial ganglia and the hindbrain are poorly formed in the *Wnt1-3a* double and the β-catenin mutant. Arrows point to the abnormal mass formed by the roots of the trigeminal ganglion (white) and the combined superior ganglion of nerves VII and VIII (black). Arrowheads in E to H indicate the position of the first DRG, absent in G,H. Asterisks show the next three DRGs, which partially remain in the β-catenin mutant. V, trigeminal ganglion; VII and VIII, combined ganglion of facial and vestibulocochlear nerves; IX, glossopharyngeal nerve; X, vagus nerve; XII, hypoglossal nerve.

8C,D) and no apoptotic cells were detected here. At 10.5 dpc (Fig. 8E,F), increased apoptosis was observed in the frontonasal mass of mutant embryos (large arrowhead in Fig. 8F) and in proximal parts of branchial arches 1 and 2, at the site where chondrogenic condensation usually occurs (small arrowheads in Fig. 8F), possibly accounting for the absence of craniofacial structures at 18.5 dpc.

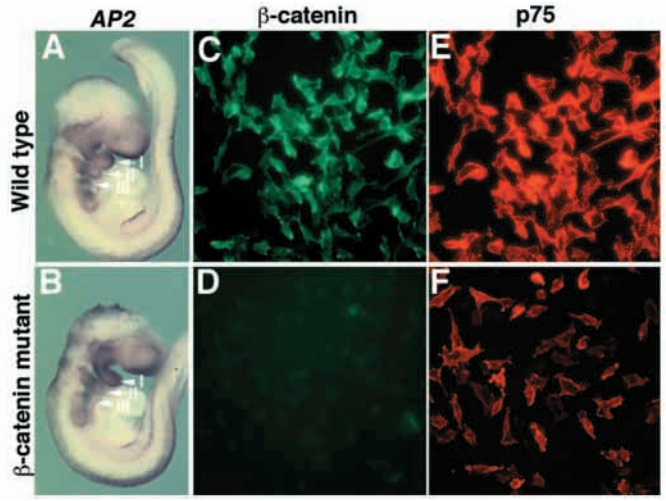

Fig. 7. Elimination of β-catenin in NCCs by *Wnt1-Cre*. The NCC marker, AP2, is expressed in streams of cells emigrating from r2 and r4 to branchial arches 1 (I) and 2 (II) by in situ hybridization in both wild-type (A) and mutant (B) embryos, indicating NCC migration to the branchial arches in β-catenin mutant embryos. Efficient elimination of β-catenin by *Wnt1-Cre* in migratory NCCs. Double immunofluorescence of wild-type (C,E) and mutant (D,F) NCC explants with an antibody to the C terminus of β-catenin (C,D) and for NCC marker p75 (E,F) demonstrates the absence of β-catenin in p75-positive NCCs in the β-catenin mutant.

DISCUSSION

β-Catenin and brain morphogenesis

The genes for Wnts, cadherins and catenins are expressed widely in the developing CNS, but mRNAs accumulate in very specific patterns as development proceeds (Roelink and Nusse, 1991; Shimamura and Takeichi, 1992; Parr et al., 1993; Hollyday et al., 1995; Redies and Takeichi, 1996; Kimura et al., 1996; Grove et al., 1998; Yamaguchi et al., 1999; Lee et al., 2000; Redies, 2000). This suggests that both Wnt signaling and cadherin cell adhesion are involved in early brain patterning and morphogenesis. Indeed, Wnt1 controls the regional patterning of the midbrain and hindbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; McMahon et al., 1992), and the proliferation of CNS stem cells (Dickinson et al., 1994). Classical cadherins, which complex with catenins, have also been shown to function in cell sorting during neural development, and in the regionalization of the CNS (Redies, 1995; Redies and Takeichi, 1996; Redies, 2000).

The aim of the present study was to determine potential roles of β-catenin during CNS development and to ask whether the activity of Wnt1 was dependent on β-catenin signaling during midbrain development. *Wnt1*$^{-/-}$ embryos lack the entire midbrain and the cerebellum, which originates from the anterior metencephalon (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). As β-catenin mutants also lack part of the midbrain and anterior hindbrain, this provides strong evidence that Wnt1 directs midbrain-hindbrain development via β-catenin signaling. Wnt1 signaling in the midbrain is required for maintenance of *En1*-expressing cells of the midbrain and anterior hindbrain (McMahon et al., 1992), and

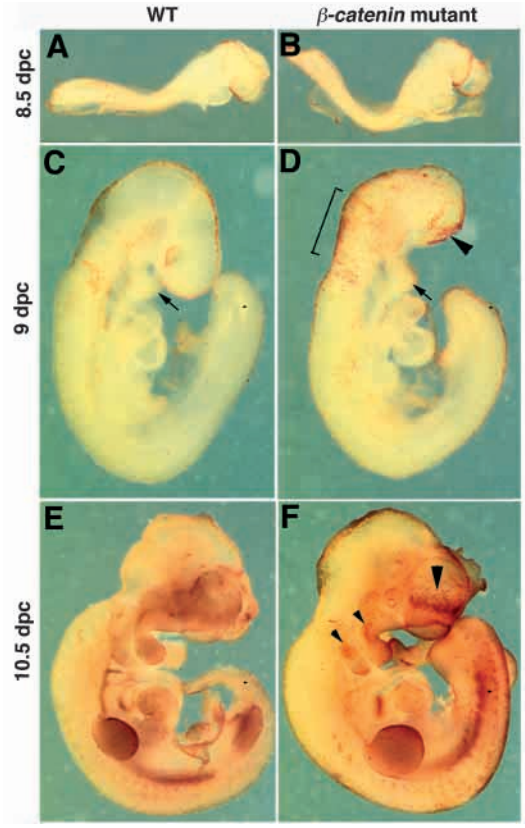

Fig. 8. Apoptosis in migratory NCCs. Wild-type (A,C,E) and β-catenin mutant (B,D,F) embryos were analyzed by TUNEL. At 8.5 dpc (A versus B), no increased apoptosis was seen in NCCs migrating to the branchial arches. At 9 dpc (C versus D), however, increased apoptosis was visible in the mutant in the frontonasal mass (arrowhead) as well as in NCCs migrating to the cranial ganglia (brackets). The branchial arches (arrows) at this stage look normal in the mutant, with no increase in cell death. At 10.5 dpc (E versus F), there is massive apoptosis in the frontonasal mass of the β-catenin mutant (big arrowhead) and increased apoptosis is observed in the proximal parts of branchial arches 1 and 2 (small arrowheads).

expression of *En1* under a *Wnt1* regulatory element in *Wnt1*$^{-/-}$ embryos resulted in substantial rescue of midbrain-hindbrain morphogenesis (Danielian and McMahon, 1996). Interestingly, inactivation of β-catenin function in the midbrain also leads to the absence of *En1* expression, providing good evidence that Wnt1/β-catenin signaling controls *En1* expression in embryonic midbrain.

Some heterogeneity was observed in β-catenin mutant embryos in the extent of midbrain and hindbrain deletion, with areas of midbrain tissue occasionally present, although devoid of neurogenesis. These residual tissues also showed expression of certain midbrain and rostral hindbrain markers, e.g. *Fgf8* and *Wnt1*. The most likely explanation for the apparent discrepancy between the *Wnt1*$^{-/-}$ *and* β-catenin mutant phenotypes is mosaic expression of *Wnt1* (Bally-Cuif et al., 1995) and hence of the *Cre* transgene in the presumptive midbrain. Wnt1 secreted to neighboring cells that did not express *Wnt1* or *Cre*, and hence would not have deleted the gene for β-catenin, could still be transducing Wnt1 signaling, but only leading to a very thin epithelium that is unable to develop into a normal midbrain. Moreover, the broad expression of *Wnt1* across the presumptive midbrain occurs for a very short period of time, possibly too short to enable all cells expressing *Wnt1* to efficiently delete the β-catenin gene. Alternatively, Wnt1 signaling may have been transduced before Cre is functional, resulting in an expansion of neural precursors, and only later, with the onset of Cre activity, would Wnt1/β-catenin function in midbrain patterning and/or cell survival be abolished. Also, removal of β-catenin only affects Wnt signaling in cells expressing *Wnt1*, whereas removal of *Wnt1* has potential long-range effects, owing to its additional paracrine signaling, adding several layers of complexity in comparing the two phenotypes.

Histological analysis at 12.5 dpc in the β-catenin mutant shows an additional absence of the choroid plexus, the boundary between metencephalon and myelencephalon. This suggests that the hindbrain deletion might extend more posteriorly into r2. Perhaps overlapping Wnt3a and Wnt3 signaling in r2 contributes to choroid plexus formation and also depends on β-catenin function. β-catenin mutants exhibit a strong phenotype in the forebrain not seen in *Wnt1*$^{-/-}$ embryos. Although up to 9.5 dpc the forebrain appears normal as judged by the expression of *Otx2* and *Fgf8*, as well as *Wnt7b* (data not shown), at 10.5 dpc it is enlarged and the walls of the telencephalic vesicles look thinner. Remarkably, increased apoptosis is observed in this region. This loss of forebrain structures also occurs in *Wnt1-3a* double mutant embryos (Ikeya et al., 1997; S. Lee, M. I. and A. P. M., unpublished), suggesting that β-catenin is required in the developing forebrain for transducing signals from Wnt3a and/or other Wnts. Thus, removal of β-catenin could circumvent the potential redundancy of the different *Wnts* expressed in the CNS, revealing some hidden functions of Wnt signaling. The loss of forebrain structures could also be a consequence of reduced NCC migration to this region. Such a view is supported by experiments in which removal of NCCs from the anterior head of chick embryos affected forebrain viability (Etchevers et al., 1999). Alternatively, the forebrain phenotype in the β-catenin mutant could be due to the lack of β-catenin function in cadherin-mediated adhesion. Many cadherins are differentially expressed in the developing CNS (Redies and Takeichi, 1996; Takeichi et al., 1997; Gerhardt et al., 2000; Redies et al., 2000) and the lack of β-catenin could perturb cadherin-mediated adhesion in CNS morphogenesis.

β-catenin and NCCs

Cranial NCCs contribute extensively to forming craniofacial structures. They migrate into the first branchial arch from the midbrain and anterior hindbrain at around the four-somite stage (Nichols, 1981; Tan and Morriss-Kay, 1986; Serbedzija et al., 1992; Chai et al., 1998). These cells will form the skeleton of the upper and lower jaw, and contribute to the trigeminal ganglion (Noden, 1978; Le Douarin, 1984; Tan and Morriss-Kay, 1985). These cranial NCC-derived structures are absent in the β-catenin mutant. Early loss of midbrain cannot account for the absence of NCCs generated in this region because skeletal structures derived from this region are unaffected in either *Wnt1*$^{-/-}$ or *Wnt1-3a* double mutants (McMahon and Bradley, 1990; Ikeya et al., 1997). *Wnt1* expression occurs in the progenitors of migrating NCCs derived from the dorsal CNS. By using the *Wnt1* regulatory sequences to drive *Cre*

expression, the β-catenin gene is permanently deleted in these cells and their descendants. This is best documented by the NCC explant cultures where β-catenin is efficiently eliminated in all NCC precursors. The lack of β-catenin is thus likely to affect NCC differentiation and survival.

Both the β-catenin and *Wnt1-3a* double mutants have defects in the formation of the cranial ganglia and DRGs, suggesting that not only Wnt1, but also Wnt3a, acts via a β-catenin-dependent pathway. Defects in DRGs are less severe in the β-catenin mutant, perhaps because Wnt1 and/or Wnt3a have time to signal before β-catenin is lost. Indeed the β-catenin mutant has no body axis truncation caudal to the forelimbs as observed in both *Wnt3a*$^{-/-}$ *and Wnt1-3a* double mutants (Takada et al., 1994; Ikeya et al., 1997). This less severe phenotype could alternatively be due to the paracrine action of Wnt signaling whereas the deletion of the gene for β-catenin restricts the effect of Wnt signaling to the *Wnt1*-expressing cells via autocrine signaling. Conversely, the cranial ganglia are more affected in the β-catenin mutant, possibly via effects on signaling by other Wnts, e.g. Wnt4 and Wnt3a, again invoking the potential of deleting the β-catenin gene to overcome redundancy of Wnts. Massive apoptosis is detected in the β-catenin mutant in areas where NCCs migrate to the cranial ganglia (Fig. 8D), suggesting that β-catenin is required for the survival of migrating NCCs. Whether this increased cell death is also due to altered cadherin-mediated cell adhesion remains unknown.

Analysis of skeletal preparations of 18.5 dpc β-catenin mutant fetuses reveals that mainly cranial bones and cartilages of cranial NCC origin are missing. The absence of craniofacial structures appears not to result from a perturbed migration of cranial NCCs, as the expression of several NCC markers was normal. Also, mutant NCCs migrated in neural tube explant cultures. Up to 9 dpc, no increased apoptosis in early NCCs migrating to the branchial arches was observed, and no apoptotic cells could be detected in branchial arches. However, starting at 10.5 dpc, apoptosis was seen in the β-catenin mutant in proximal parts of arches 1 and 2, at the position where chondrogenic condensations take place. Condensations are cellular products of epithelial-mesenchymal cell interactions that initiate cell differentiation and morphogenesis within the branchial arches (Le Douarin, 1982; Carlson, 1994). Condensations require intimate cell-cell contact and hence recruitment of molecules mediating cell-cell adhesion. We therefore propose that the absence of craniofacial development in the β-catenin mutant is due to perturbation of cadherin-mediated cell adhesion rather than to a defect in Wnt signaling.

In conclusion, we have shown that β-catenin is required for brain morphogenesis and for forming craniofacial structures derived from NCCs. The β-catenin mutant phenotype largely resembles the *Wnt1*$^{-/-}$ phenotype, i.e. in lacking midbrain/hindbrain structures, indicating that Wnt1/β-catenin signaling is required in these developmental processes. Moreover, our results provide good evidence that the control of *En1* expression by Wnt1 is mediated through β-catenin-dependent signaling. We further observe a rather strong phenotype in craniofacial structures not seen in *Wnt1*$^{-/-}$ embryos. It is likely that here the lack of β-catenin affects cadherin function. Clearly, a more specific gene inactivation scheme is required to dissect the functions of β-catenin in adhesion vs. signaling.

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