A splice variant of the Wilms' tumour suppressor *Wt1* is required for normal development of the olfactory system

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Summary

Neuronal lineage formation in the developing olfactory epithelium has been extensively studied at the cellular level, but little is known about the genes that control proliferation and differentiation of neuronal progenitor cells. Here, we report that the Wilms' tumour zinc-finger protein, Wt1, is required for normal formation of the olfactory epithelium. Wt1 was detected by immunohistochemistry in the developing olfactory epithelium of wild-type embryos between gestational days E9.5 and E18.5. Embryos with complete lack of *Wt1* and embryos with selective ablation of the alternatively spliced *Wt1(+KTS)* isoform both had thinner olfactory epithelia and fewer neuronal progenitor cells than do normal animals. Mash1 and neurogenin 1, two basic helix-loop-helix transcription factors with critical functions during olfactory neuron development, were reduced in the *Wt1(+KTS)*$^{-/-}$ mutants compared with the wild-type mice. Stable expression of the *Wt1(+KTS)* isoform, but not of the *Wt1(–KTS)* variant, upregulated Mash1 mRNA and protein in vitro. The olfactory epithelia of mouse embryos, which lacked the Wt1(–KTS) protein, appeared normal. However, formation of the neural retina was severely impaired in the *Wt1(–KTS)*$^{-/-}$ mutants. These findings demonstrate that the Wt1(+KTS) protein, which has been proposed to play a role in mRNA processing, acts upstream of Mash1 to promote the development of the olfactory epithelium. Furthermore, neuron formation depends on distinct functions of alternatively spliced *Wt1* products in the embryonic retina and the olfactory epithelium.

Key words: Wt1, Olfactory epithelium, Mash1, Alternative splicing, Neuron development

Introduction

The Wilms' tumour gene, *Wt1*, encodes a zinc-finger protein, which is required for normal embryonic development. Mouse embryos with homozygous *Wt1* defects (*Wt1*$^{-/-}$) die in utero and exhibit a failure of normal formation of the kidneys (Kreidberg et al., 1993), gonads (Kreidberg et al., 1993), spleen (Herzer et al., 1999), adrenal glands (Moore et al., 1999) and mesothelial tissues (Kreidberg et al., 1993; Moore et al., 1999). We have recently found that *Wt1* is also required at different stages of development of the retina. Retinal defects in the *Wt1*$^{-/-}$ mutants were characterized by an impaired proliferation of neuronal progenitor cells and apoptotic loss of a large fraction of ganglion cell precursors (Wagner et al., 2002a). Previous reports on Wt1 expression in distinct regions of the CNS, including ependymal cells of the spinal cord and the area postrema in the brain (Armstrong et al., 1992; Sharma et al., 1992; Rackley et al., 1993), suggested an even wider role for *Wt1* in the formation of neuronal tissues. In support of these morphological studies, we found that inhibition of Wt1 with antisense oligonucleotides abolished the potential of human retinoblastoma cells to undergo neuronal differentiation in vitro (Wagner et al., 2002b). However, is it not known whether loss of Wt1 leads to defects in the developing CNS in addition to altered retina formation, nor is it understood how Wt1 fulfils its proposed functions during neuron development in molecular terms.

At least 24 different Wt1 proteins are generated by the combination of alternative mRNA splicing (Haber et al., 1991; Gessler et al., 1992), the use of variable translation start sites (Bruening and Pelletier, 1996; Scharnhorst et al., 1999), and RNA editing (Sharma et al., 1994). Among the various gene products, alternatively spliced exon 5 encodes 17 amino acids, and the use of two alternative splice donor sites at the end of exon 9 leads to the insertion/omission of a tripeptide (lysine-threonine-serine, KTS) between zinc fingers 3 and 4 of the Wt1 molecule (Haber et al., 1991). The proteins, which are encoded by the alternatively spliced *Wt1* forms, are designated as Wt1(–KTS) and Wt1(+KTS), respectively. Wt1(–KTS) has been reported to function as both, an activator and a repressor of gene transcription (Englert et al., 1995a; Lee et al., 1999) (reviewed by Menke et al., 1998; Scharnhorst et al., 2001). By contrast, the results of several studies suggested that the +KTS isoforms, which comprise more than 50% of the Wt1 proteins (Haber et al., 1991; Hammes et al., 2001), could play a role in mRNA processing (Larsson et al., 1995; Englert et al., 1995b;

Ladomery et al., 1999). Thus, the *Wt1(+KTS)* products colocalized with and bound to the nuclear splicing factor U2AF65 (Davies et al., 1998). Moreover, computer modelling (Kennedy et al., 1996) and in vitro studies (Caricasole et al., 1996) testified that the +KTS proteins bind to RNA, whereas the –KTS isoforms preferentially interact with DNA sequences. However, bona fide downstream targets of the Wt1(+KTS) products have not been identified yet. In an effort to analyse the roles of different Wt1 proteins during development, mouse lines with selective ablation of either of the two splice insertions were generated. Although removal of exon 5 caused no obvious phenotypic abnormalities (Natoli et al., 2002), selective ablation either of the *Wt1(–KTS)* or the *Wt1(+KTS)* product revealed distinct functions of these proteins during gonad and kidney formation (Hammes et al., 2001). The specific roles of the –KTS and +KTS proteins, which are conserved among vertebrates (Kent et al., 1995; Miles et al., 1998), have not been analysed in neuronal tissues yet.

The present study served a twofold purpose. First, we aimed to further establish a role for Wt1 in neuronal development through identifying novel sites of Wt1 expression in the immature CNS. Second, by comparing the phenotype of mouse embryos with selective inactivation either of the *–KTS* or the *+KTS* variant, we made a first step towards understanding specific functions of alternatively spliced *Wt1* gene products in the developing brain.

Materials and methods

Animals

A detailed description of the generation of mutant mice with specific lack either of the *Wt1(+KTS)* or the *Wt1(–KTS)* splice variant, is given elsewhere (Hammes et al., 2001). Genotyping of the embryos was performed by PCR analysis of genomic DNA according to our previous protocol (Hammes et al., 2001).

Histology and immunohistochemistry

Morphological studies were performed as described in detail elsewhere (Wagner et al., 2002a; Wagner et al., 2003). Staged embryos (morning of vaginal plug was considered E0.5) were fixed overnight at 4°C in paraformaldehyde (3% in PBS) and either embedded in paraffin wax for Haematoxylin-Eosin (HE) staining or snap-frozen in pre-chilled isopentane and then embedded in Tissue-Tek® OCT compound (Sakura Finetek, Netherlands) for immunohistochemical analyses. Tissue sections (10 μm) were cut and transferred onto gelatin-coated glass slides. The tissue sections were permeabilized with 0.1% Triton X-100 in PBS and blocked by incubation for 1 hour in 10% normal serum (in PBS, 0.1% Triton X-100, 3% BSA), which was obtained from the same species as the secondary antibody. Following treatment (16 hours, 4°C) with primary antibody and 3×15 minutes washes in PBS, the slides were incubated for 1.5 hours with biotinylated secondary antibodies (1:150 dilutions in PBS, 1% BSA, Vector Laboratories) and streptavidin-Cy3 complex (Sigma, Deisenhofen, Germany). The sections were viewed under an epifluorescence microscope (Axiovert S100, Zeiss, Jena, Germany), which was connected to a digital camera (Spot RT Slider, Diagnostic Instruments), using the Metamorph V4.1.2 software (Universal Imaging). For double immunostaining, the first antigen was detected using the Vector M.O.M. immunodetection kit (Vector Laboratories) and streptavidin-Cy3 complex followed by incubation with the second primary antibody and a Cy2-labelled secondary antibody. Appropriate negative controls were made using normal sera instead of primary antibodies. The following primary antibodies were used for immunohistochemical analyses: Wt1 polyclonal antibody from rabbit diluted 1:150 (C-19, sc-192, Santa Cruz Biotechnology, Heidelberg, Germany), Wt1 monoclonal antibody from mouse diluted 1:100 (clone 6F-H2, MAB4234, Chemicon), Pou4f1 mouse monoclonal antibody diluted 1:150 (14A6, sc-8429, Santa Cruz Biotechnology), Pou4f2 polyclonal antibody from rabbit diluted 1:150 (C-13, sc-6026, Santa Cruz Biotechnology), Ki-67 polyclonal antibody from goat diluted 1:150 (M-19, sc-7846, Santa Cruz Biotechnology), neurogenin 1 polyclonal antibody from rabbit diluted 1:150 (AB5680, Chemicon, Temecula, CA), Mash1 rabbit polyclonal antibody diluted 1:150 (AB5696, Chemicon), Mash1 monoclonal antibody from mouse diluted 1:100 (clone 24B72D11.1, 556604, BD Biosciences), NCAM polyclonal antibody from rabbit diluted 1:1000 (AB5032, Chemicon) and GFAP polyclonal antibody from rabbit diluted 1:1000 (AB5804, Chemicon).

Detection of apoptotic cells

Apoptotic cells were localized in the olfactory epithelia of paraformaldehyde-fixed mouse embryos by TUNEL-labelling with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) as described in detail previously (Wagner et al., 2002a). Five 10 μm transverse sections of the olfactory epithelium were obtained from each animal to mark the apoptotic cells. Five animals were studied in each group at E18.5.

Cell culture

The human embryonic kidney cell line, HEK293 (ATCC CRL-1573), was purchased from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% FCS (Biochrom KG, Berlin, Germany), 100 IU/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen). The cells were split twice per week at ~80% confluence for routine maintenance. The transfection procedure and the selection of clones with stable expression of the Wt1(–KTS) and Wt1(+KTS) proteins is described elsewhere (Wagner et al., 2001).

Reverse transcription (RT) PCR

Total RNA was prepared from HEK293 cells using the Trizol reagent (Invitrogen). The RNA pellet was dissolved in diethyl pyrocarbonate-treated H_2O at a concentration of 1 μg/μl. First-strand cDNA synthesis was performed with 2 μg of total RNA using oligo(dT) primers and superscript II reverse transcriptase (Invitrogen). One-tenth of the reaction product was used for PCR amplification in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer) according to the following protocol: DNA denaturation at 94°C, primer annealing at 58°C, extension of double-stranded DNA at 72°C (32 cycles, each step lasting 30 seconds). The following primers were used for PCR amplification: human *GAPDH*, 5′-AACAGCGACACCCACTCCTC-3′ (forward primer) and 5′-GGAGGGGAGATTCAGTGTGGT-3′ (reverse primer); human achaete-scute complex-like 1 (*ASCL1*), 5′-GAACTGATGCGCTGCAAACGC-3′ (forward primer) and 5′-CGGCCATGGAGTTCAAGTCGT-3′ (reverse primer); mouse *Wt1*, 5′-ATCAGATGAACCTAGGAG-3′ (forward primer) and 5′-CTGGGTATGCACACATGA-3′ (reverse primer). The amplified DNA sequences were 257 bp (*GAPDH*), 333 bp (*ASCL1*) and 269 bp (*Wt1*) long.

SDS-PAGE

Total cell lysates from subconfluent cultures of HEK293 cells were prepared in a buffer consisting of 8 M urea, 10% (v/v) glycerol, 1% SDS, 10 mM Tris, pH 6.8 supplemented with 1× protease inhibitor cocktail (Roche Molecular Biochemicals), 10 mM DTT and 1 mM vanadate. Protein (60 μg) was heated to 95°C for 3 minutes in Laemmli buffer (500 mM Tris-HCl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) and run on a 10% polyacrylaminde gel. The separated proteins were transferred onto

polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany) with the use of a semidry blotting apparatus (BioRad, München, Germany). Non-specific binding was reduced by incubating the membranes for 60 minutes at room temperature in PBS, 5% Blotto (Santa Cruz Biotechnology), 0.05% Tween-20 (Serva, Heidelberg, Germany). Incubation with a polyclonal anti-Wt1 antibody from rabbit (C-19, sc-846, Santa Cruz Biotechnology, 1:100 dilution in PBS, 5% Blotto, 0.05% Tween-20) and polyclonal anti-Mash1 antibody from goat (C-16, sc-13222, Santa Cruz Biotechnology, 1:100 dilution in PBS, 5% Blotto, 0.05% Tween-20) was performed overnight at 4°C. After 3×15 minutes washes in PBS, 0.05% Tween-20, incubation was performed at room temperature for 1 hour either with peroxidase-coupled goat anti-rabbit secondary antibody to detect Wt1 or with rabbit anti-goat secondary antibody to detect Mash1 (1:1.000 dilution in PBS, 5% Blotto, 0.05% Tween-20). Following 3×15 minutes washes in PBS, 0.05% Tween-20, the reaction products were detected with the enhanced chemoluminescence system (Amersham Pharmacia Biotech, Freiburg, Germany). For further analysis, the blots were stripped with 0.2 M glycine, pH 2.5, at 56°C for 30 minutes and reprobed with a goat polyclonal antibody against β-actin (1:500 dilution in PBS, 5% Blotto, 0.05% Tween-20, C-11, sc-1615, Santa Cruz Biotechnology).

Results

Wt1 is expressed in the developing olfactory epithelium of mice

In addition to the known sites of Wt1 expression in the developing brain (Armstrong et al., 1992; Sharma et al., 1992; Rackley et al., 1993), we detected Wt1 by immunohistochemistry and in situ mRNA hybridisation in the olfactory epithelium of normal mouse embryos. Wt1 was identified in the nasal placode at embryonic day 9.5 (E9.5), the earliest time point studied (not shown). Cells with nuclear Wt1 staining were located predominantly in the basal region of the developing olfactory epithelium at E18.5 (Fig. 1). We used double-immunofluorescent labelling of the neural cell adhesion molecule, NCAM (Calof and Chikaraishi, 1989; Key and Akeson, 1990), to determine whether the Wt1-expressing cells in the developing olfactory epithelium were committed to the neuronal lineage. Evidently, a proportion of the Wt1-positive cells in the sensory olfactory epithelium also reacted with anti-NCAM antibody indicating their neuronal cell fate (Fig. 1). In contrast, Wt1 expression did not overlap with the glial fibrillary acidic protein (GFAP) (Fig. 1).

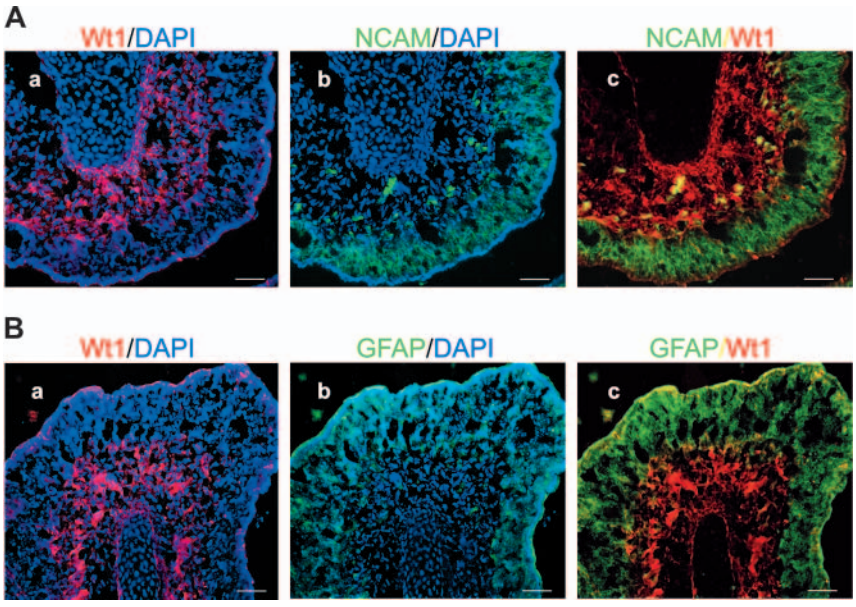

Fig. 1. Immunostaining of Wt1, neural cell adhesion molecule (NCAM) and glial fibrillary acidic protein (GFAP) in the olfactory epithelium of a wild-type mouse embryo at E18.5. Wt1 was detected in the nuclei of a significant proportion of cells at the base of the olfactory epithelium (a). Double-immunostaining of NCAM (green fluorescence) and Wt1 (red fluorescence) revealed an overlapping pattern of both proteins (Ac). By contrast, Wt1 and glial fibrillary acidic protein (GFAP) were not co-localized in cells of the olfactory epithelium (B). The results shown are representative for the more than 20 tissue sections from five different embryos. Scale bars: 50 μm.

Olfactory development is impaired in embryos with lack of the *Wt1(+KTS)* splice variant

To examine whether *Wt1* is required for normal formation of the olfactory system, we compared the sensory epithelia of wild-type and $Wt1^{-/-}$ mutant embryos at E12.5. Strikingly, olfactory development was impaired in the *Wt1*-deficient animals, whose sensory epithelium was markedly thinner than that of normal embryos (Fig. 2). For comparison, the vomeronasal organ – a chemosensory neuroepithelium that does not express Wt1 (K.D.W. and N.W., unpublished) – developed normally in $Wt1^{-/-}$ embryos (Fig. 2E,F) indicating that there is no general delay in neuronal differentiation in $Wt1^{-/-}$ mutants.

We have shown recently that two alternatively spliced *Wt1* forms, which can be distinguished by the insertion/omission of three amino acids (lysine-threonine-serine, KTS) between the third and fourth zinc finger, have distinct roles in genitourinary development (Hammes et al., 2001). To explore whether the Wt1(+KTS) and Wt1(–KTS) proteins also exert different functions during the formation of neuronal tissues, we analysed the forebrains of mouse embryos with specific lack of either Wt1(+KTS) or Wt1(–KTS). Like in mice with complete disruption of *Wt1*, the olfactory epithelium was clearly thinner in the *Wt1(+KTS)*-deficient embryos than in wild-type animals at E12.5 and E18.5 (Fig. 3). By contrast, the olfactory epithelium seemed normal in mouse embryos, which lacked the *Wt1(–KTS)* isoform (Fig. 3). Embryos with heterozygous defects either of the +KTS or the –KTS variant had a normal olfactory epithelium, which could not be distinguished from that of wild-type mice (not shown).

Axon fibres, which are formed by the olfactory receptor neurons project to the olfactory bulb, where they form synaptic connections with other neurons. Normal differentiation of the olfactory bulb depends on the incoming signals from the sensory neurons. As a consequence, impaired formation of the olfactory epithelium in the *Wt1(+KTS)*-deficient embryos may affect olfactory bulb development. To further assess the role of Wt1 in the development of the olfactory system, we studied the morphology of olfactory bulbs in wild-type and *Wt1*-mutant

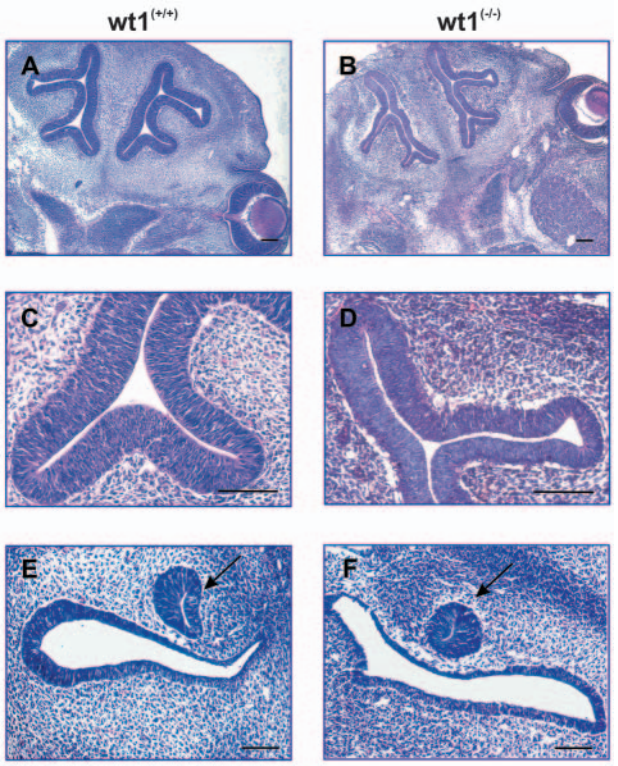

Fig. 2. Haematoxylin-Eosin (HE) staining of horizontal tissue sections through the forebrain of E12.5 wild-type (*Wt1*$^{+/+}$) embryos and embryos with complete disruption of *Wt1* (*Wt1*$^{-/-}$). (A-D) The olfactory epithelium is thinner in the *Wt1*-deficient than in the the *Wt1*$^{+/+}$ embryo. (E,F) The vomeronasal organs (arrows) were normally developed in the *Wt1*$^{-/-}$ embryos. The tissue sections are representative for the three different animals of each group analysed. Scale bars: 100 μm.

embryos. Compared with normal (*Wt1*$^{+/+}$) and *Wt1(–KTS)*-deficient animals, the sizes of the olfactory bulbs were markedly reduced in E18.5 embryos, which lacked the *Wt1(+KTS)* protein (Fig. 3). Although the typical layered architecture of the olfactory bulb was maintained in the *Wt1(+KTS)*$^{-/-}$ mutants, the cells were clearly fewer in the olfactory bulbs of *Wt1(+KTS)*$^{-/-}$ than in normal and *Wt1(–KTS)*-deficient embryos (Fig. 3). Notably, Wt1 could not be detected by immunohistochemistry in the olfactory bulbs of wild-type embryos at any time point studied (not shown).

Next, we addressed the question whether abnormal development of the olfactory epithelium in the *Wt1(+KTS)*$^{-/-}$ mutant mice was due to reduced cell survival and/or impaired proliferation of neuronal progenitor cells. A total of six tissue sections from five different E18.5 embryos in each group of the wild-type as well as the *Wt1(+KTS)*$^{-/-}$ and the *Wt1(–KTS)*$^{-/-}$ mice were analysed. The numbers of TUNEL-positive cells that were identified in the basal parts of the olfactory epithelium on each tissue slide (Fig. 4) were 32±7 in wild-type animals, 65±7 in the *Wt1(–KTS)*$^{-/-}$ mutants and 167±4 in embryos with lack of the *Wt1(+KTS)* product (ANOVA Test with Dunn post-hoc test, wild-type versus the *Wt1(+KTS)*$^{-/-}$ mutants, $P<0.05$). Furthermore, the number of Ki-67-positive cells was reduced in the olfactory epithelia of *Wt1(+KTS)*-deficient embryos compared with the wild-type and *Wt1(–KTS)*$^{-/-}$ mice at E18.5 (Fig. 4). Similarly, immunostaining of the proliferating cell nuclear antigen (PCNA) was weaker in mouse embryos with specific lack of the Wt1(+KTS) protein (not shown).

Mash1 and neurogenin 1 are reduced in the olfactory epithelium with lack of Wt1(+KTS)

Immunolabelling was used to reveal potential downstream mediators of Wt1(+KTS) in the developing olfactory epithelium. The mammalian homologue of *achaete-scute* complex, *Mash1* (*Ascl1* – Mouse Genome Informatics), is among the molecules that are required during the early stages of olfactory epithelium formation. *Mash1* encodes a proneural basic helix-loop-helix (bHLH) transcription factor, whose inactivation in mice severely reduced the number of olfactory progenitor cells (Guillemot et al., 1993; Cau et al., 2002). We performed immunofluorescent labelling to identify Mash1-expressing cells in a total of six tissue sections from five different animals each at E18.5. Mash1 was readily detected in a significant number of cells in the basal region of the olfactory epithelium in both, wild-type and *Wt1(–KTS)*-deficient embryos (Fig. 5). By contrast, Mash1 immunoreactivity was reduced dramatically in the olfactory epithelium of mouse embryos with lack of the Wt1(+KTS) protein (Fig. 5). Furthermore, the bHLH transcription factor neurogenin 1, whose expression is activated by Mash1 (Cau et al., 1997), could barely be identified in the olfactory sensory epithelium of the *Wt1(+KTS)*$^{-/-}$ mutant embryos. For comparison, the neuronal transcription factor Pou4f1 (formerly Brn3a) was still detectable, though in fewer cells, in the olfactory epithelium of E18.5 embryos with lack of Wt1(+KTS) (Fig. 5).

Mash1 is upregulated in cells with stable expression of the Wt1(+KTS) protein

To investigate whether *Mash1* expression is regulated by the Wt1(+KTS) protein, we made use of our previously established human embryonic kidney (HEK) 293 cell lines. These are clones with stable expression either of the *Wt1(–KTS)* or the *Wt1(+KTS)* isoform (Wagner et al., 2001). HEK293 cells were originally isolated from primary human embryonic kidney cells transformed by sheared adenovirus 5 DNA (Graham et al., 1977). Recent findings indicate that HEK293 cells are related to neurons rather than renal epithelial cells, which could make them a suitable model for studying neuronal gene regulation (Shaw et al., 2002). *Mash1* transcripts were hardly detectable by RT-PCR in HEK293 cells, which had been transfected with the empty expression vector (Fig. 6). Although forced expression of the *Wt1(–KTS)* splice variant produced only a slight increase in *Mash1* transcripts, mRNA levels were clearly elevated in the *Wt1(+KTS)* transfected cells (Fig. 6). Upregulation of Mash1 in these cells was also demonstrated at the protein level by immunoblotting with a polyclonal anti-Mash1 antibody (Fig. 6).

Reporter gene assays were performed to investigate whether the promoter of the Mash1 gene could be stimulated by the different Wt1 forms. For this purpose, we transiently co-transfected HEK293 cells with a luciferase reporter, which contained a 1436 bp fragment of the predicted human achaete-scute complex homolog-like (*ASCL1*) gene promoter sequence (NCBI number U77616) together with *Wt1(+KTS)* and *Wt1(–KTS)* expression constructs. Notably, co-transfection

neither of the *Wt1(–KTS)* nor of the *Wt1(+KTS)* expression construct significantly enhanced the activity of the *Mash1*-luciferase reporter (not shown). Accordingly, we also cloned a 1132 bp fragment, which carried the predicted promoter of the murine *Mash1* gene (Ensembl gene ENSMUSG00000020052). Similar to the findings with the human *ASCL1* regulatory

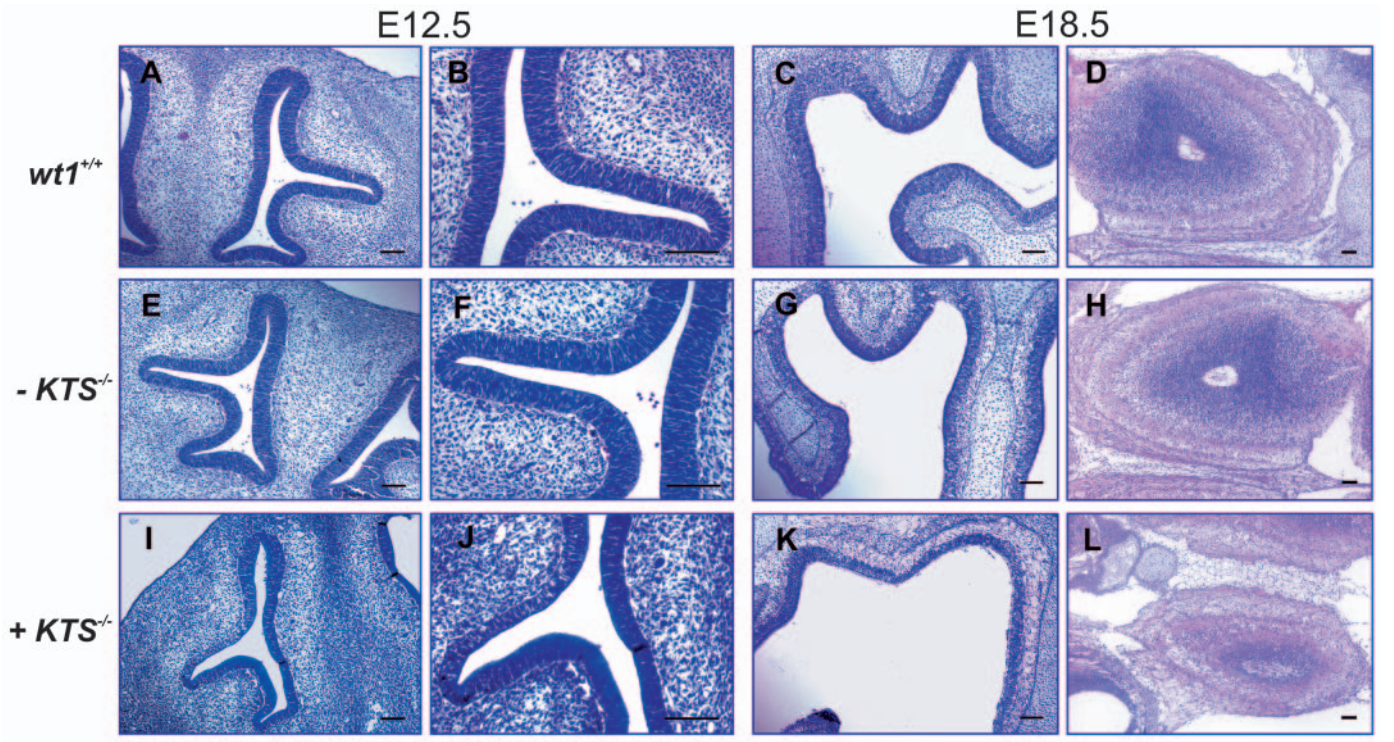

Fig. 3. HE-staining of horizontal tissue sections through the olfactory epithelia and olfactory bulbs of mouse embryos at E12.5 and E18.5. The sections were cut from wild-type embryos ($Wt1^{+/+}$) and from age-matched mice with specific lack either of the *Wt1(–KTS)* or the *Wt1(+KTS)* splice variant. Strikingly, the *Wt1(+KTS)*-deficient embryos (I-K) exhibited thinner olfactory epithelia than wild-type animals (A-C) and embryos with lack of the Wt1(–KTS) protein (E-G). (D,H,L) The olfactory bulb in the *Wt1(+KTS)*-deficient (L) embryo is reduced in size and hypocellular compared with the wild-type embryos (D). Olfactory bulb morphology appeared normal in embryos with inactivation of *Wt1(–KTS)* (H). Representative data for five embryos from each group are shown. Scale bars: 100 μm.

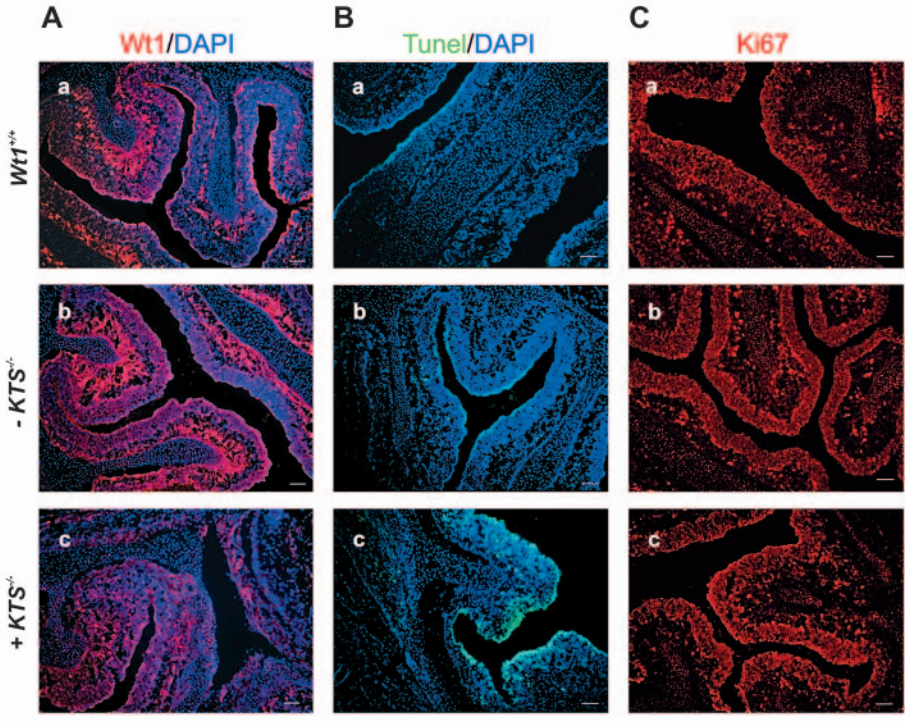

Fig. 4. Immunostaining of Wt1 (A), TUNEL-labelling of apoptotic cells (B) and Ki-67 staining of proliferating cells (C) in the olfactory epithelium of a wild-type embryo at E18.5 and in age-matched embryos with splice-specific *Wt1* defects. The results shown are representative of the more than 20 tissue sections that were obtained from five animals in each group. (B) More TUNEL-positive (apoptotic) cells were present in the olfactory epithelia of embryos with lack of the +KTS isoform than in wild-type and *Wt1(–KTS)*-deficient mice. (C) The number of Ki-67 positive cells was slightly reduced in mouse embryos with lack of Wt1(+KTS) compared with normal and Wt1(–KTS)-deficient embryos. Scale bars: 100 μm.

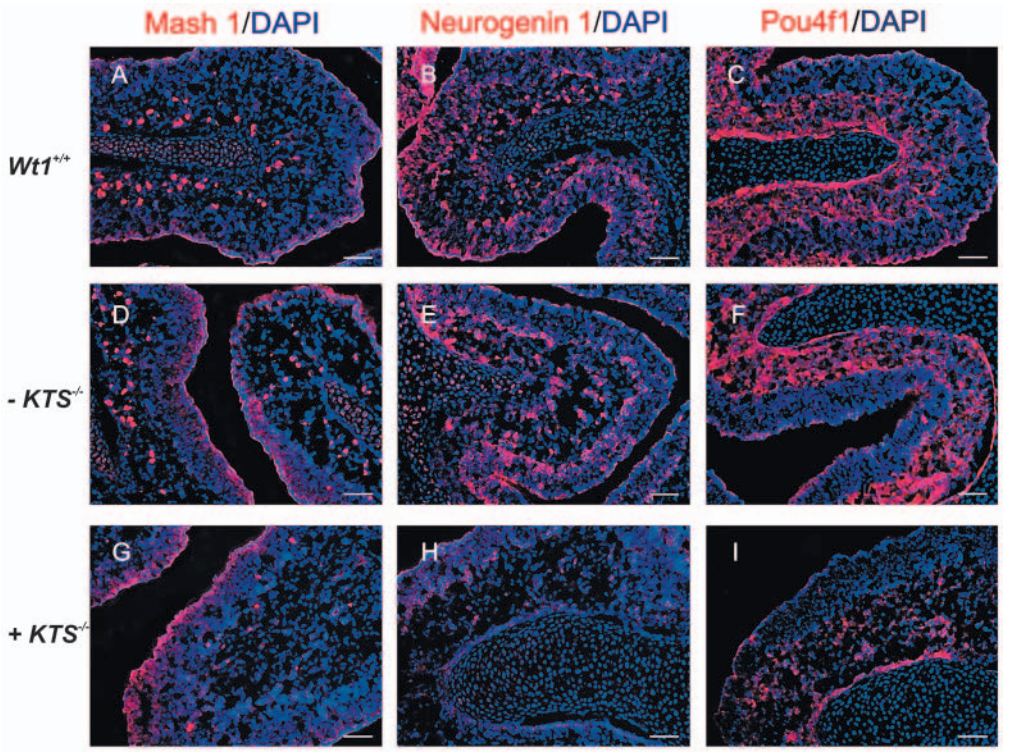

Fig. 5. Immunostaining of proneural transcription factors in the olfactory epithelia of normal embryos at E18.5 (A-C), and of mice with inactivation either of the *Wt1(–KTS)* (D-F) or the *Wt1(+KTS)* (G-I) splice variant. Expression of the mammalian homologue of *achaete-scute* complex, *Mash1* (*Ascl1*), was reduced in the olfactory epithelia of *Wt1(+KTS)*-deficient mice (G). The basic helix-loop-helix transcription factor neurogenin1, which is activated by Mash1 (Cau et al., 1997), was detected in fewer olfactory epithelial cells in embryos with lack of the *Wt1(+KTS)* product (H) compared with wild-type (B) and *Wt1(–KTS)*-deficient (E) mice. By comparison, only subtle differences in the expression of Pou4f1 were detectable between the different groups (E,F,I). Scale bars: 50 μm.

sequence, the *Wt1(–KTS)* and *Wt1(+KTS)* products did not stimulate transcription from the murine *Mash1* promoter, although this sequence contained two predicted *Wt1(+KTS)*-binding sites (not shown).

A double-immunofluorescent staining procedure was applied to explore whether Wt1 and Mash1 are colocalized in cells of the developing olfactory epithelium. Remarkably, a proportion of cells expressed both proteins suggesting that Wt1 can possibly regulate *Mash1* expression also in the developing olfactory epithelium in vivo (Fig. 6).

Different *Wt1* splice variants are predominant during development of the retina and the olfactory epithelium

We have recently found that *Wt1* is necessary for normal development of the retina (Wagner et al., 2002a). Complete inactivation of *Wt1* caused severe retinal defects consisting in an impaired proliferation of neuronal progenitor cells and an apoptotic loss of ganglion cell precursors (Wagner et al., 2002a). To distinguish which of the alternatively spliced Wt1 variants would be crucial for the development of the

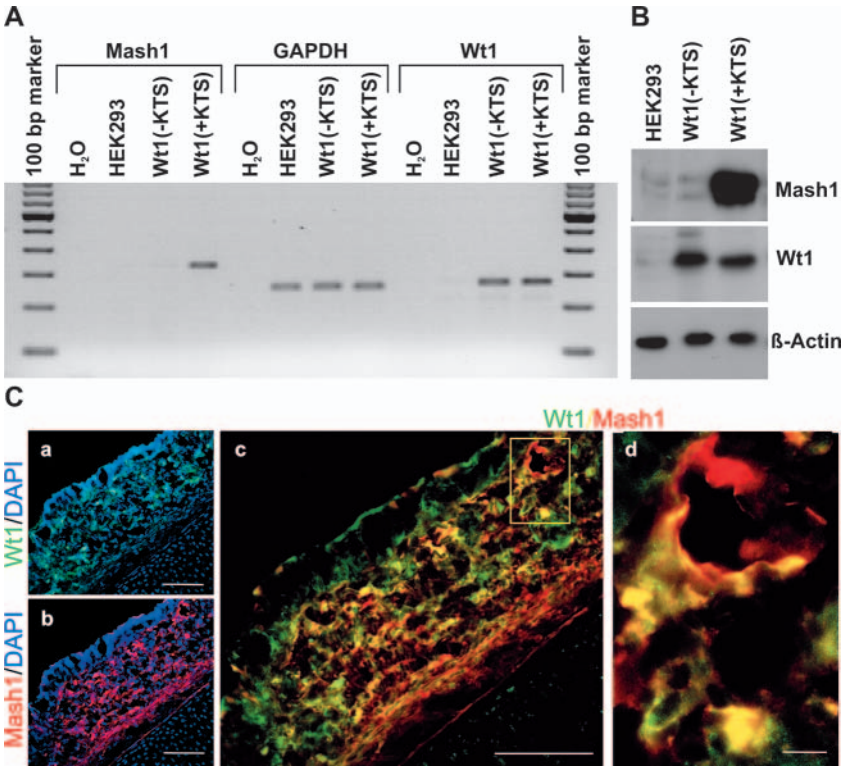

Fig. 6. Mash1 mRNA (A) and protein (B) in human embryonic kidney (HEK) 293 cells, which had been stably transfected either with empty expression vector (HEK293) or with the *Wt1(–KTS)* and *Wt1(+KTS)* splice variants, respectively. (A) *Mash1*, *GAPDH* and *Wt1* transcripts were detected by reverse transcription PCR. Data shown are representative for the three independent clones that were analysed. Stable expression of Wt1(+KTS), but not of the –KTS variant, induced *Mash1* mRNA in HEK293 cells. (B) Stimulation of Mash1 by the *Wt1(+KTS)* product was confirmed by immunoblotting with a polyclonal anti-Mash1 antibody. (C) A partially overlapping pattern of Wt1 (green) and Mash1 (red) was revealed by double-immunostaining in cells of the developing olfactory epithelium (E18.5). Scale bars: 100 μm in C, parts a, b, c; 10 μm in C, part d.

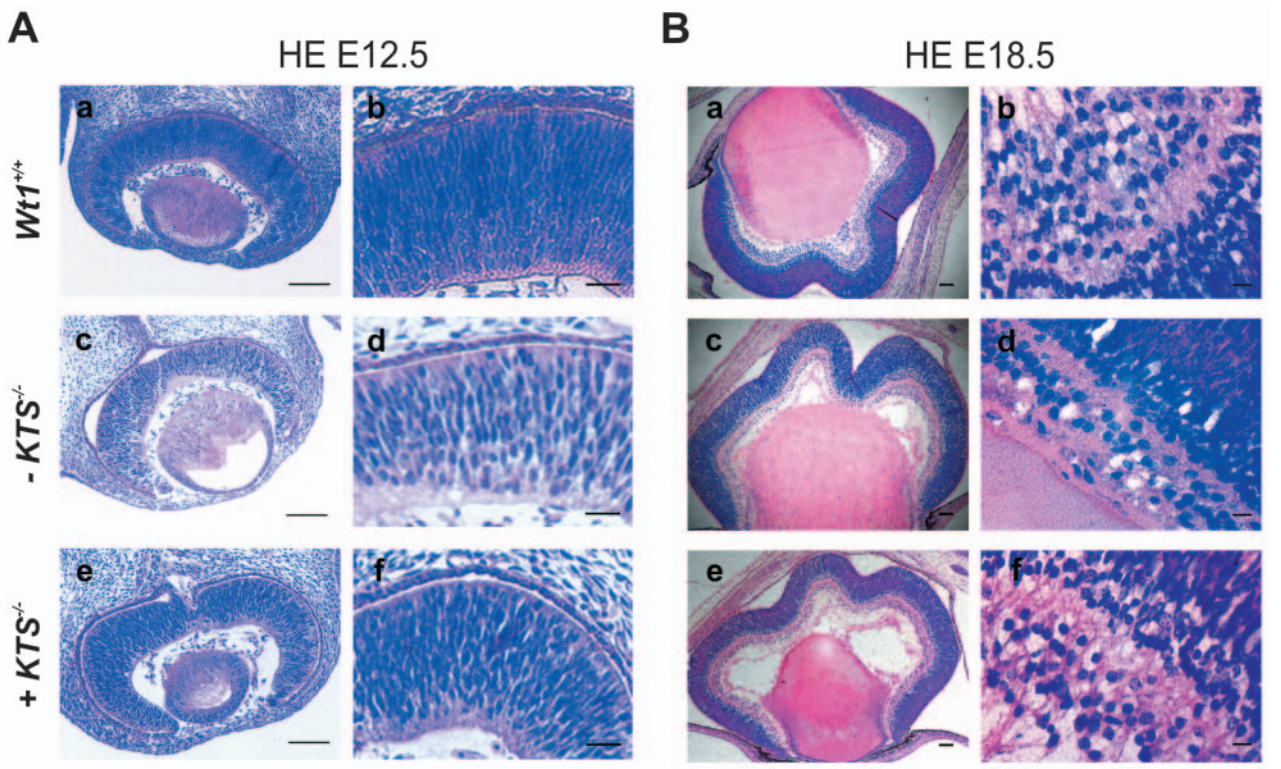

Fig. 7. Representative HE-staining of the developing eyes of mouse embryos at E12.5 (A) and E18.5 (B). Strikingly, the *Wt1(–KTS)*-deficient embryos exhibited thinner retinas with fewer cells (A, parts c, d) than age-matched normal mice (A, parts a, b). For comparison, the ocular phenotype was less severe in embryos with inactivated *Wt1(+KTS)* (A, parts e, f). (B) Abnormal morphology of the retina, in particular the reduced cell density in the future ganglion cell layer, was clearly evident in *Wt1(–KTS)*-deficient embryos at E18.5 (part d; compare with b and f). Scale bars: 10 µm in B, parts b, d, f; 50 µm in A, parts b, d, f; 50 µm in B, parts a, c, e; 100 µm in A, parts a, c, e.

neural retina, we analysed the ocular phenotype of mice with specific lack either of the Wt1(–KTS) or the Wt1(+KTS) protein. Compared with the wild-type embryos at E12.5, the retinas of the *Wt1(–KTS)*$^{-/-}$ mutants were clearly thinner and contained fewer cells (Fig. 7A). Abnormalities of the developing ganglion cell layer became visible at E18.5 in the *Wt1(–KTS)*-deficient embryos (Fig. 7B). These retinal defects were less severe in embryos with lack of *Wt1(+KTS)* (Fig. 7), indicating that normal formation of the retina depends mainly on the function of the Wt1(–KTS) protein.

Our recent findings indicate that Wt1(–KTS) is a transcriptional activator of the *Pou4f2* gene (Wagner et al., 2003). *Pou4f2* (formerly *Brn3b*) encodes a proneural transcription factor, which is required for retinal ganglion cell survival and optic nerve fibre growth (Gan et al., 1996; Erkman et al., 1996). Hence, it has been shown that Pou4f2 acts a downstream mediator of Wt1 in the immature retina (Wagner et al., 2003). This finding is supported by our present results, showing that immunostaining of Pou4f2 was weaker in the future ganglion cell layer of embryos (E18.5) with lack of Wt1(–KTS) than in the wild-type and Wt1(+KTS)$^{-/-}$ mice (Fig. 8).

Discussion

The olfactory sensory epithelium is unique among neuronal tissues because of its ability to continuously regenerate throughout adulthood (Moulton, 1974; Grazaidei et al., 1978). It can therefore serve as a model system with which to elucidate the molecular mechanisms of neurogenesis, which may eventually allow one to manipulate the potential regenerative capacity of nerve cells. Previous findings have suggested that similar mechanisms might be responsible for the differentiation of adult stem cells and the formation of neurons in the developing olfactory epithelium (Parras et al., 2004) (reviewed by Mackay-Sim and Chuah, 2000).

Induction of the olfactory sensory tissue, which originates from ectodermally derived neurogenic placodes, is dependent on mesenchymal/epithelial interaction in the developing forebrain (LaMantia et al., 2000). Among the molecules that have been implicated in the epithelial conversion of mesenchymal cells in other organs is the product of the Wilms' tumour gene, *Wt1* (Kreidberg et al., 1993; Moore et al., 1999). *Wt1* was originally identified by its mutational inactivation in a subgroup of paediatric renal tumours (Wilms' tumours, nephroblastomas) (reviewed by Hastie, 1994). Subsequent studies revealed a crucial role for *Wt1* in the formation of the genitourinary system (Kreidberg et al., 1993) and other epithelial tissues of mesenchymal origin (Herzer et al., 1999; Moore et al., 1999). We have recently discovered that Wt1 is also crucial for neurogenesis, in that the retinas of *Wt1*-deficient mice failed to develop normally (Wagner et al., 2002a). Our current findings extend the role of *Wt1* in neuronal differentiation by demonstrating that the formation of the olfactory epithelium is severely disturbed in *Wt1(+KTS)*-deficient mice. Although the initial formation of the olfactory

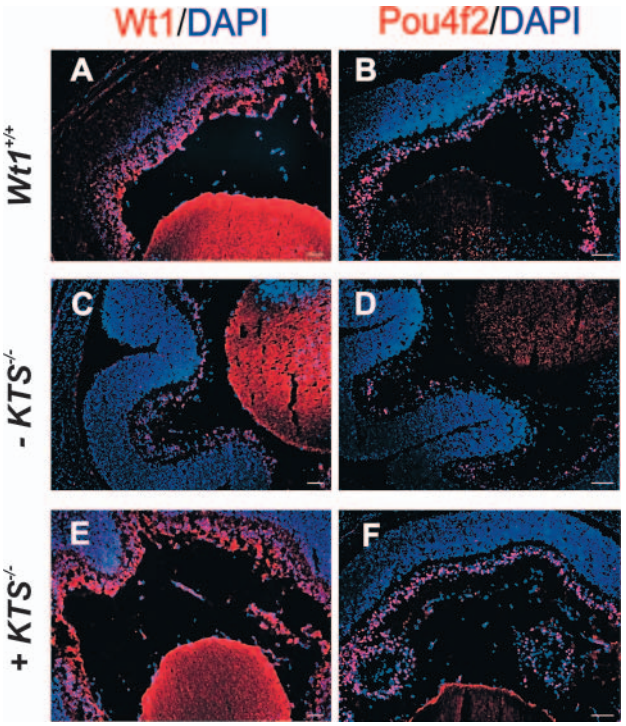

Fig. 8. Immunofluorescent labelling of Wt1 and Pou4f2 in the developing retinas of mouse embryos at E18.5. Loss of retinal ganglion cells in mice with lack of *Wt1(–KTS)* (C,D) is reflected in the reduction of Wt1- and Pou4f2-positive cells compared with normal ($Wt1^{+/+}$; A,B) and *Wt1(+KTS)*-deficient (E,F) embryos. Notably, the class IV POU domain factor, Pou4f2 (formerly Brn3b), was recently identified as a transcriptional target of Wt1(–KTS) (Wagner et al., 2002a; Wagner et al., 2003). Scale bars: 50 μm.

sensory tissue in the *Wt1(+KTS)* mutants seemed intact, defective morphology at E12.5 indicates a requirement for *Wt1* during the early stages of olfactory development. A reduced proliferation of progenitor cells of the *Wt1(+KTS)*-deficient embryos is suggested from the fewer Ki-67 positive cells in the olfactory epithelium compared with wild-type and $Wt1(–KTS)^{–/–}$ mutant mice. In addition, more TUNEL-positive cells were detected in the olfactory epithelia of mice, which lacked the *Wt1(+KTS)* form, than in normal and *Wt1(–KTS)*-deficient embryos. Thus, enhanced apoptotic cell death may account, at least in part, for the defective olfactory epithelia of embryos with inactivation of *Wt1(+KTS)*. Very similar observations have previously been made in the developing neuronal retina of mice with disrupted *Wt1* gene, which also displayed more TUNEL-positive cells than wild-type embryos (Wagner et al., 2002a), and in the kidneys and gonads of *Wt1*-deficient mice (Kreidberg et al., 1993; Hammes et al., 2001). Thus, apoptotic cell death in $Wt1^{–/–}$ embryos appears to occur mainly in tissues that would express Wt1 in normal mice. It remains to be established whether Wt1 rescues cells from apoptosis through a direct anti-apoptotic action. Alternatively, and perhaps even more likely, Wt1 may function as a regulator of cell differentiation, and lack of Wt1 will lead to apoptosis due to a failure of normal cellular specification.

Altered formation of the olfactory epithelium in embryos with ablation of the Wt1(+KTS) protein, is also reflected in their hypoplastic olfactory bulbs. Notably, Wt1 could not be detected, at least by the means of immunohistochemistry, in the olfactory bulbs of wild-type embryos. Consequently, impaired olfactory bulb formation in the *Wt1(+KTS)*-deficient embryos was secondary to their abnormal olfactory epithelium rather than resulting from a cell-autonomous defect of olfactory bulb cells. Interestingly, defects of the developing olfactory system became apparent only in embryos, which lacked the *Wt1(+KTS)* splice variant, but not in the $Wt1(–KTS)^{–/–}$ mutants. However, embryos with ablation of *Wt1(–KTS)* had more severe retinal abnormalities than the *Wt1(+KTS)*-deficient mice. Similar to embryos with complete *Wt1* knockout (Wagner et al., 2002a), the $Wt1(–KTS)^{–/–}$ retinas contained fewer progenitor cells in addition to their failure to form a normal ganglion cell layer. Thus, the development of the retina seems to depend mainly on the function of the Wt1(–KTS) protein, which has been implicated in the control of gene transcription. Accordingly, the Pou-domain factor Pou4f2 (formerly Brn-3b), which is required for retinal ganglion cell development (Gan et al., 1996; Erkman et al., 1996) and whose transcription is activated by Wt1(–KTS) (Wagner et al., 2003), was virtually missing in the ganglion cells of the $Wt1(–KTS)^{–/–}$ retinas.

Evidence has been provided that Wt1 proteins, which contain the +KTS splice insertion, might act at a post-transcriptional level rather than functioning as transcriptional regulators (Larsson et al., 1995; Englert et al., 1995b; Davies et al., 1998; Ladomery et al., 1999; Laity et al., 2000). However, physiologically relevant in vivo targets of the Wt1(+KTS) isoforms have not been identified yet. By comparing the gene expression profiles in the olfactory epithelia of normal embryos and of mice with lack of the *Wt1(+KTS)* variant, one may eventually succeed in isolating potential downstream target molecules. A first candidate gene for regulation by the Wt1(+KTS) protein could be the mammalian homologue of *achaete-scute* complex, *Mash1* (*Ascl1*). Expression of *Mash1*, which encodes a basic helix-loop-helix (bHLH) transcription factor, was reduced in the *Wt1(+KTS)*-deficient olfactory epithelium. Mash1 is a crucial molecule for the proliferation and neuronal specification of progenitor cells in the ventral telencephalon (Casarosa et al., 1999). Mice with homozygous null alleles for *Mash1* exhibited a severe reduction of olfactory neurons due to impaired progenitor cell proliferation and apoptotic cell death (Guillemot et al., 1993; Cau et al., 1997). Neurogenin 1, another proneural bHLH transcription factor, is expressed at a later stage of olfactory progenitor development than Mash1 (Cau et al., 1997). Remarkably, most cells in the olfactory epithelium of *Mash1*-null mutant embryos failed to produce neurogenin 1, indicating that Mash1 is required for normal expression of neurogenin 1 (Cau et al., 1997). Consistently, we found that both proteins, Mash1 and neurogenin 1, were only weakly expressed in the olfactory epithelia of *Wt1(+KTS)*-deficient embryos. It remains to be clarified whether the *Wt1(+KTS)* splice product provides a signal for the proliferation and/or survival of Mash1-positive olfactory progenitor cells, or whether it may even stimulate the expression of Mash1 more directly. The latter possibility is supported by our observation that Mash1 was enhanced by forced expression of Wt1(+KTS), but not of the –KTS variant, in cultured cells derived from human embryonic kidney.

Recent findings suggest that the HEK293 cells, which we used, resemble neurons rather than renal epithelial cells (Shaw et al., 2002). Their neuronal origin, which is indicated by the expression of several neuron-specific marker proteins in HEK293 cells (Shaw et al., 2002), could be a reason for the strong increase of Mash1 in response to forced Wt1 expression. The molecular mechanism by which Wt1(+KTS) activates the expression of *Mash1* remains to be further clarified in future studies. The lack of stimulation of the Mash1 promoter by Wt1 argues in favour of either a post-transcriptional interaction between Wt1(+KTS) and *Mash1*, or simply signifies that additional cis-regulatory elements, which were not contained in our promoter construct, are required. Remarkably, a significant fraction of Wt1-immunopositve cells in the developing olfactory epithelium of wild-type embryos also contained Mash1. This observation points to the possibility that Wt1 can activate the expression of Mash1 not only in cultured cells, but also in neuronal progenitor cells in vivo. Taken together, our findings demonstrate that a splice variant of the Wilms' tumour gene *Wt1* plays a crucial role during development of the olfactory system. The phenotype of mouse embryos with lack of the *Wt1(+KTS)* product reveals a requirement of this protein for the proliferation and survival of olfactory progenitor cells. On the contrary, formation of the neuronal retina mainly depends on the Wt1(–KTS) protein, which acts as a transcription factor. In conclusion, neuron formation in the embryonic retina and the olfactory epithelium requires different functions exerted by alternatively spliced *Wt1* products.

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