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FGFR1 regulated gene-expression, cell proliferation and differentiation in the developing midbrain and hindbrain

Tomi Jukkola

Institute of Biotechnology and
Department of Biological and Environmental Sciences
Division of Genetics
Faculty of Biosciences and
Helsinki Graduate School of Biotechnology and Molecular Biology
University of Helsinki
Finland

Academic dissertation

To be presented, with the permission of the Faculty of Biosciences of the University of Helsinki, for public criticism, in the auditorium 1041 at the Viikki Biocenter 2, Viikinkaari 5, on August 17th 2007, at 12 o’clock noon.
If we knew what we were doing we wouldn’t call it science.

Albert Einstein
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptophan (serotonin)</td>
</tr>
<tr>
<td>ANR</td>
<td>anterior neural ridge</td>
</tr>
<tr>
<td>AP</td>
<td>anterioposterior</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatosis polyposis coli protein</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic factor</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>Cb</td>
<td>cerebellum</td>
</tr>
<tr>
<td>CNPY1</td>
<td>canopy1</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cp</td>
<td>choroid plexus</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>cRNA</td>
<td>synthetic complementary RNA to mRNA produced from a DNA template</td>
</tr>
<tr>
<td>DA</td>
<td>dopaminergic</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamino-2-phenylindole</td>
</tr>
<tr>
<td>Di</td>
<td>diencephalon</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DRAPC1</td>
<td>adenomatosis polyposis coli down-regulated 1, APCDD1 protein</td>
</tr>
<tr>
<td>DV</td>
<td>dorsoventral</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EN</td>
<td>engrailed homeobox transcription factor</td>
</tr>
<tr>
<td>ERM (or ETV5)</td>
<td>member of the PEA3 group of ETS domain containing TFs</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FLRT</td>
<td>family of glycosylated proteins containing a fibronectin III domain and leucine rich repeats (LRR) domain</td>
</tr>
<tr>
<td>FST</td>
<td>follistatin protein</td>
</tr>
<tr>
<td>FZ</td>
<td>frizzleds which are members of the seven transmembrane domain cell surface receptors</td>
</tr>
<tr>
<td>GBX2</td>
<td>gastrulation brain homeobox 2 transcription factor</td>
</tr>
<tr>
<td>GRG4 (or TLE4)</td>
<td>groucho-related gene, transducin-like enhancer of split 4</td>
</tr>
<tr>
<td>HH</td>
<td>Hamburger Hamilton stage</td>
</tr>
<tr>
<td>HOX</td>
<td>a particular subgroup of homeobox transcription factors</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulphate</td>
</tr>
<tr>
<td>Ic</td>
<td>inferior colliculus</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IsO</td>
<td>isthmic organizer</td>
</tr>
</tbody>
</table>
kDa kilo Dalton (molecular weight)
KO knockout
LC locus coeruleus
MAPK mitogen activated protein kinase
Mb midbrain
MHB midbrain–hindbrain boundary
MKP3 MAPK phosphatase-3
mRNA messenger ribonucleic acid
MRP multi-drug resistance protein
OTX orthodenticle homolog (Drosophila)
p21 (or Cdkn1a) 21 kDa cyclin dependent kinase inhibiting protein
PAX paired domain containing homeobox transcription factor
PCR polymerase chain reaction
PH3 phosphohistone H3
PI3K phosphatidylinositol 3’ kinase
R (e.g. R1) rhombomere (e.g. rhombomere 1)
RA retinoic acid
RNA ribonucleic acid
RrF retrorubral field
SA serotonergic
Sc superior colliculus
SEF similar expression to Fgfs
SFRP secreted frizzled-related protein
SHH sonic hedgehog protein
siRNA small interfering RNA used to decrease transcription
SNC substantia nigra pars compacta
SOX SRY-related HMG-box transcription factor
SPRYs sprouty family of proteins
Te telencephalon
Teg tegmentum
TF(s) transcription factor(s)
TH tyrosine hydroxylase
TRH thyrotrophin releasing hormone
TUJ neuron specific beta III Tubulin
TUNEL terminal deoxynucleotidyl transferase mediated nick labeling
Tyr tyrosine residue
v vermis of the cerebellum
VTA ventral tegmental area
VZ ventricular zone
WNT(1) wingless-related MMTV integration site 1
ZLI zona limitans intrathalamica
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following three articles and one manuscript as well as unpublished data. In the text they are referred to by their roman numerals.


*equal contribution
SUMMARY

The neuroectodermal tissue close to the midbrain-hindbrain boundary (MHB) is an important secondary organizer in the developing neural tube. This so-called isthmic organizer (IsO) regulates cellular survival, patterning and proliferation in the midbrain (Mb) and rhombomere 1 (R1) of the hindbrain. Signaling molecules of the IsO, such as fibroblast growth factor 8 (FGF8) and WNT1 are expressed in distinct bands of cells around the MHB. It has been previously shown that FGF-receptor 1 (FGFR1) is required for the normal development of this brain region in the mouse embryo. In the present study, we have compared the gene expression profiles of wild-type and Fgfr1 mutant embryos. We show that the loss of Fgfr1 results in the downregulation of several genes expressed close to the MHB and in the disappearance of gene expression gradients in the midbrain and R1. Our microarray screen identified several previously uncharacterized genes which may participate in the development of midbrain–R1 region. Our results also show altered neurogenesis in the midbrain and R1 of the Fgfr1 mutants. Interestingly, the neuronal progenitors in midbrain and R1 show different responses to the loss of signaling through FGFR1. As Wnt1 expression at the MHB region requires the FGF signaling pathway, WNT target genes, including Drapc1, were also identified in our screen. The microarray data analysis also suggested that the cells next to the midbrain-hindbrain boundary express distinct cell cycle regulators. We showed that the cells close to the border appeared to have unique features. These cells proliferate less rapidly than the surrounding cells. Unlike the cells further away from the boundary, these cells express Fgfr1 but not the other FGF receptors. The slowly proliferating boundary cells are necessary for development of the characteristic isthmic constriction. They may also contribute to compartmentalization of this brain region.
REVIEW OF THE LITERATURE

1. The early brain development

1.1. Neural induction and neurulation

The complex development of central nervous system (CNS) begins already prior to the gastrulation. The CNS begins as a flattened layer of neuroepithelial cells on the dorsal surface of the embryo. They are induced by the signals from the surrounding ectoderm and mesenchyme. The molecular interactions create a sheet of cells that are forming the neural plate. With the onset of neurulation, the embryo also begins to elongate along its anterioposterior (AP) axis. At the anterior end of the primitive streak of the gastrulating mouse embryo (approximately at embryonic (E) day 7.5) lays an important structure, the node. The inductive and organizing activity of an early node was first reported in amphibians by Spemann and Mangold (reviewed in Hamburger, 1988). In these experiments the dorsal blastoporal lip from a newt gastrula stage embryo was transplanted into another embryo, which induced the formation of a secondary embryo with a normal body pattern. The inductive and organizing activity of an early node was first reported in amphibians by Spemann and Mangold (reviewed in Hamburger, 1988). In these experiments the dorsal blastoporal lip from a newt gastrula stage embryo was transplanted into another embryo, which induced the formation of a secondary embryo with a normal body pattern. The inductive properties of the transplanted tissue indicated that the node (or Spemann’s organizer in amphibians) not only instructed cells in adjacent tissues to acquire new fates, but also provided information about the development of AP axis. The inductive properties of the transplanted tissue indicated that the node (or Spemann’s organizer in amphibians) not only instructed cells in adjacent tissues to acquire new fates, but also provided information about the development of AP axis. 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During neurulation the lateral edges of the plate, or neural folds, become prominent and begin to bend toward each other to form a tube. As the neural folds of the plate close, the dorsal midline of the embryo begins to sink somewhat below the surface, and in making contact, the central depression in the plate becomes more distinctive. As they become adjacent, the neural folds fuse, producing a hollow neural tube and a continuous dorsal epithelium. After separation from the overlying epithelium, the neural tube thickens differentially as various regions begin to form different subregions of the brain and spinal cord.

1.2. Molecular events of neural induction and neurulation

Recent research has identified specific molecules that bring about neural induction. The complex set of molecular interactions commits ectodermal cells over the notochord to becoming neural tissue, being the first step in the formation of the nervous system. In amphibians, three secreted molecules, noggin (Nog), follistatin (Fst), and chordin (Chrd), are among the inductive proteins. It was first thought that these molecules directly protect the ectoderm from epidermalizing signals and protect the mesoderm from ventralizing signals, allowing the development of a patterned neural plate (Harland and Gerhart, 1997). However, subsequent research on amphibians has shown that these inductors act by blocking the action of an inhibitors, such as bone morphogenetic protein 4 (BMP4), in the dorsal ectoderm (reviewed in Lee and Jessell, 1999). Subsequently, in the absence of BMP4 activity, dorsal ectoderm forms neural tissue as a default state. In
amphibians, neural specification requires the continuous activity of various BMP antagonists from blastula through gastrula stages. Due to redundancy of different BMP antagonists only the inactivation of three of them (Nog, Fst, and Chrd) has been shown to result to a disruption of dorsal development (Khokha et al., 2005). In birds and mammals, the inactivation of BMPs is also likely important in neural induction, but the role of noggin, follistatin and chordin as inhibitors of BMPs is less clear. For example, mice that lack Fst (Matzuk et al., 1995) or Nog (McMahon et al., 1998) function do not show obvious defects in neural induction although later neural defects are observed in Nog-/- mice. In addition, in mouse, Bmp4 mutant embryos do not exhibit an apparent expansion in neural tissue, as might have been anticipated from experiments of BMP4 inactivation in amphibians (Winnier et al., 1995).

In addition to BMP antagonists, other mechanisms have been demonstrated to decrease the BMP signaling in neural induction. For example, in the chick, early FGF expression downregulates Bmp expression in the prospective neural plate (Wilson et al., 2000). FGF8 signaling is also able to initiate the events of neural induction whereas addition of noggin or chordin to the chick epiblast can alter the position of the neural folds but cannot induce ectopic neural tissues (Streit et al., 2000).

2. Mechanisms of brain patterning

2.1. Anterioposterior patterning

An important second step is initial regionalization of the CNS into broad anterioposterior regions. Numerous experiments have shown that tissue explants of the early primitive node can induce a nervous system with both anterior (head) and posterior (trunk) components, whereas explants of older node tissue only induce trunk characteristics (reviewed in Harland and Gerhart, 1997). Earlier experiments also showed that certain inductors cause the formation of more anterior, and others more posterior, neural structures. For example, in the presence of retinoic acid (RA), WNTs, or FGFs, the induced neural structures are posteriorized, and more caudal structures form (Kudoh et al., 2002; Storey et al., 1998). In mammals, the anterior hypoblast (or anterior visceral endoderm (AVE) in mouse) expresses genes characteristic of the prechordal plate and probably initiates head formation (Stern, 2001). Shortly following the neural induction, additional signals from the notochord and AVE result in the expression of the transcription factor Otx2 in the head region (forebrain/midbrain) and Gbx2 in the trunk region (hindbrain/spinal cord). Both the signaling molecules and transcription factors are important for the anterior structures. To produce a head region, it is necessary to block signals from the RA/WNT/FGF signaling pathways as well as the BMP4 signal.

2.2. Isthmic organizer

The proper development of the CNS requires a fine-tuned pattern of cell identities along the AP-axis. Further specification and compartmentalization of the different neuronal domains is guided by so called secondary signaling centers (Fig. 1A). The isthmic organizer (IsO) is such a center which is formed between midbrain (Mb) and the most anterior part of the hindbrain, rhombomere 1 (R1).
Figure 1. The role of signaling molecules and transcription factors in the development of midbrain-R1 region. (A) Side view of an E10.5 mouse embryo. (B) Dorsal view of an E10.5 embryo. (C) Midsagittal view of an E12.5 mouse embryo. (D) Midsagittal view of an E18.5 brain. Two secreted factors, FGF8 and WNT1, are required for the development of midbrain-R1 region (A,B). In addition to FGF8 and WNT1, Shh expression in the ventral midline regulates dorsoventral patterning and the expression of Fgf8 at midbrain-R1 region during early development (E8.5-E12)., Otx2 and Gbx2 are transcription factors which act antagonistically to set up the position of the IsO (B). The expression of the En1/En2 transcription factors are required for the maintenance of the IsO (B). The expression of the En1/En2 transcription factors are required for the induction of the Fgf8 in the IsO (Ye et al., 1998). In addition to the AP and DV patterning, the IsO signals regulate the development of neuronal population such as dopaminergic (DA) neurons in the midbrain and serotonergic (SA) neurons in the R1. Initially, bone morphogenetic proteins (BMPs) expressed in the roof plate and signals from the IsO are regulating the induction of the locus coeruleus (LC) progenitors. The development of III and IV cranial ganglia are also dependent on the IsO signaling. The IsO activity is also required for the ordered specification of inferior and superior colliculi in the dorsal midbrain (mesencephalic structures) as well as for the development of cerebellum in the dorsal R1 (metencephalic structure) (C,D). III, third cranial ganglion (or oculomotor nerve); IV, fourth cranial ganglia (or trochlear nerve); Cb, cerebellum; cp, choroid plexus; Di, diencephalon; Ic, inferior colliculus; mb, midbrain; r1, rhombomere1; Sc, superior colliculus; Te, telencephalon; Teg, tegmentum; vHb, ventral hindbrain. Adapted from Wurst and Bally-Cuif (2001) and Ang (2006).
axial position (Echevarria et al., 2003; Liu and Joyner, 2001a; Lumsden and Krumlauf, 1996; Wurst and Bally-Cuif, 2001). Transplantation of tissue grafts originating from the IsO into the caudal forebrain or hindbrain lead to the induction of ectopic midbrain or cerebellar structures, respectively (Martinez et al., 1991). Thus, the IsO seems to possess the organizing properties which are guiding the development of adjacent brain regions (see Figs. 1 and 2).

The molecular specification of isthmic cell fates appears to depend on signaling molecules that control the pattern and identity of adjacent cell types. Members of the fibroblast growth factor (FGF) family of secreted proteins have a prominent role in AP patterning of the midbrain and R1. Additional signals involving members of the WNT, sonic hedgehog (SHH) and bone morphogenetic protein (BMP) families also contribute to the correct patterning of midbrain-R1 region (see Fig. 1A,B). These planar signals emanated from the IsO are required for the development of the midbrain and cerebellum (Fig. 1C,D).

Apart from the IsO, secondary organizers have been identified at other border regions in the vertebrate neural tube (Echevarria et al., 2003; Lumsden and Krumlauf, 1996; Wurst and Bally-Cuif, 2001). For example, the border between prethalamus and thalamus called zona limitans intrathalamica (ZLI) is an important signaling center for diencephalic AP patterning (Fig. 1A). Several signaling molecules, including SHH, WNTs and FGFs, have been shown to mediate the organizer activity of ZLI (Kiecker and Lumsden, 2005; Vieira et al., 2005). In the telencephalon, at least SHH and FGF signaling from the anterior neural ridge (ANR) region are needed for the correct patterning of anterior fates such as lens and olfactory placodes (Bailey et al., 2006; Ekker et al., 1995; Ohkubo et al., 2002). It seems that Fgf8 expression in the ANR has a similar role to Fgf8 expression in the IsO where it is required for AP patterning of the adjacent tissues (Fig. 2; see below).

**Figure 2. Inductive properties of the IsO: Tissue from the midbrain-R1 region and FGF8 soaked beads can induce ectopic midbrain and cerebellum development.** Transplantation of Mb-R1 tissues to different positions induces Engrailed (En) expression gradients and affects the AP patterning as indicated. Similar induction of En2 and ectopic structure is observed with FGF8 beads. Di, diencephalon; Mb, midbrain; R1, rhombomere 1; Te, telencephalon. Adapted from Liu and Joyner (2001a).
2.2.1. Induction and localization of isthmic organizer

The molecules needed for positioning of the IsO are the two homeobox transcription factors, Otx2 and Gbx2 which are expressed at E7.5 (0 somite stage) within the anterior and posterior neuroectoderm, respectively (Fig. 3 and 4; Ang et al., 1994). This juxtaposition is maintained through mutual antagonism, resulting in a sharp midbrain/R1 border by E9.5 with Otx2 transcripts found in the forebrain and midbrain, and Gbx2 expression maintained in rhombomeres 1 to 3 (Fig. 3; Li and Joyner, 2001). In mouse mutants for Otx2 or Gbx2 genes, cells fail to maintain the positional identities (Acampora et al., 1997; Li and Joyner, 2001; Li et al., 2002; Martinez-Barbera et al., 2001). Otx2 -/- mouse embryos die early during embryogenesis and the prospective anterior brain regions are lost (Acampora et al., 1997). Gbx2 function also appears to be necessary during the early development of neural plate. Although defects are milder than in Otx2 mutant embryos, in the absence of Gbx2 function derivatives of rhombomeres 1-3 fail to form (Wassarman et al., 1997). In addition, mice homozygous for a Gbx2 hypomorphic allele shows caudal shift in expression of Fgf8 and Fgf17 domains (Waters and Lewandoski, 2006). Otx2 (and Otx1) has also been shown to antagonize and define the Shh expression domain to ventral midbrain. The co-repressor for the antagonism on Shh expression is thought to be GRG4 (Puelles et al., 2003; Puelles et al., 2004).

Induction of engrailed transcription factor genes En1 and En2 and paired box transcription factor genes Pax2 and Pax5 is regulated by the general AP patterning process (Liu and Joyner, 2001a; Olander et al., 2006). From early-somite stages, En1, En2, Pax2 and Pax5 become expressed across the Otx2/Gbx2 border in a graded fashion (see Fig. 1B and Fig. 3; Liu and Joyner, 2001b). The expression of En1 and Pax2 is earlier, whereas that of En2 and Pax5 is later than Fgf8 expression in the midbrain-R1 region (Fig. 4; Crossley and Martin, 1995). However, neither FGF8 nor WNT1 activities are required for the initial induction of Pax2/5 and En1/2 expression (Olander et al., 2006). Later in development, however, FGF8, WNT1, EN1/2 and PAX2/5 molecules...
Figure 3. Schematic of the IsO induction and development.
Homeodomain transcription factors Otx2 and Gbx2 position the isthmic organizer in the neural primordium at around E7.5 (A). The co-regulation of Otx2 and Gbx2 is thought to determine the precise location of the MHB and the transcriptional regulation might be involved in the inductive events characteristic of the isthmic organizer. Once the Otx2/Gbx2 interface (positional specification) has been set up, independent signaling pathways (WNT, FGF, RA) converge in their activity to drive organizer function (B). Fibroblast growth factors secreted from the anterior R1 are necessary for, and sufficient to mimic, organizer activity in patterning the midbrain and anterior hindbrain, and are tightly controlled by feedback inhibition (e.g. OTX1 and 2 indirectly; cytosolic SPRYs directly). Numerous feedback loops maintain appropriate midbrain-R1 region and IsO gene expression (C). A network of other secreted factors such as WNT1 as well as transcription factors, including OTX2, GBX2, EN1/2, PAX2/5/8 and GRG4, further refines the expression domain and level of FGF8 at the MHB through opposing effects on PAX2 activity (C). At this stage expression boundaries of other MHB-associated genes become also more restricted. Modified from Hynes and Rosenthal (1991), Wurst and Bally-Cuif (2001), Ye et al. (1998), Raible and Brand (2004) and Guo et al. (2007) and referenced therein.
form a positive regulatory network which maintains the isthmic specific gene-expression and is necessary for the maintenance of midbrain-R1 identity (Wurst and Bally-Cuif, 2001). The mouse En1 and En2 homeobox transcription factors are expressed in a domain encompassing the posterior midbrain and anterior hindbrain (Davidson et al., 1988; Davis and Joyner, 1988). Studies in Xenopus laevis have indicated that induction of AP neural patterning might involve signals from the dorsal mesoderm to the overlying ectoderm (Ruiz i Altaba and Jessell, 1993). Work in several species suggests that signals from the anterior mesendoderm or notochord might regulate the expression of Engrailed genes in the neural plate (Ang and Rossant, 1993; Darnell and Schoenwolf, 1997; Hemmati-Brivanlou et al., 1990; Shamim et al., 1999). However, in mouse or in zebrafish which lack the notochord, AP patterning is normal (Ang et al., 1994; Klingensmith et al., 1999; Shih and Fraser, 1996; Weinstein et al., 1994).

Expression patterns of Engrailed genes in R1 coincide with parts of the neural tube that generate dorsal and ventral structures, such as the cerebellum and pons. In the midbrain, En1 and En2 activity is regulating the development of superior and inferior colliculi and ventral midbrain nuclei (see below). En1 mutants, which die on the day of birth (E18.5), lack the cerebellum and inferior colliculus (Wurst et al., 1994). By contrast, En2 mutants are viable and fertile and have minor defects in cerebellar foliation (Millen et al., 1994). In the chicken, overexpression of En1 and En2 in midbrain showed that engrafted proteins regulate the anterior-posterior polarity of the optic tectum, the homolog of the mammalian superior colliculus (Friedman and O’Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996). In addition, mouse knockout analysis has shown that En1 and En2 are required

![Figure 4. The onset of MHB-associated genes during the IsO development.](image-url)
for the maintenance of the midbrain dopaminergic (DA) neuronal population in later stages of embryonic development (Alberi et al., 2004; Simon et al., 2001; Simon et al., 2004; Simon et al., 2005).

Another level of molecular control needed for the IsO activity relies on the secreted signaling proteins. Like in many other CNS regions, the specification of neural types in midbrain-R1 region is controlled by secreted signals derived from local signaling centers (Echevarria et al., 2003; Lumsden and Krumlauf, 1996; Wurst and Bally-Cuif, 2001). FGF8/17/18 and WNT1 are signaling molecules required for the IsO activity (Figs. 1 and 3). First $Fgf8$ and $Wnt1$ are expressed broadly in the future midbrain-R1 region but later expressions boundaries get juxtaposed (Broccoli et al., 1999). The onset of $Fgf8$ expression in mouse is around 3-5 somite stage and is largely limited to the $Gbx2$ positive side of the midbrain-R1 region (Fig. 4; Crossley and Martin, 1995). At E9.0, $Fgf8$ expression is restricted to a narrow band at the most anterior part of the R1 whereas the $Wnt1$-positive cells are located in the midbrain (Wurst and Bally-Cuif, 2001). After the initiation of $Fgf8$ expression, GBX activity is maintaining the $Fgf8$ expression in the R1, whereas OTX2 and GBX2/FGF8 regulate each other negatively, leading to refinement of the sharp expression boundaries (see Fig. 3; Broccoli et al., 1999; Wassarman et al., 1997; Millet et al 1999). Initially, $Wnt1$ expression is detected broadly in the prospective midbrain territory, but its expression boundaries are also later refined (Figs. 3 and 4). At E10, $Wnt1$ expression is detected as a narrow band that surrounds the most posterior midbrain, just anterior to the $Fgf8$ expression domain. Dorsally, $Wnt1$ expression is also restricted to a narrow stripe of cells located in the roof plate (tectum). Strong $Wnt1$ expression domain is located also in the most ventral midbrain (tegmentum). In contrast to dynamic $Fgf8$ and $Wnt1$ expression, the expression of $En1$, $En2$, $Pax2$ and $Pax5$ remains relatively broad on both sides of the $Otx2/Gbx2$ border between E7.5 and E12.5.

In chicken FGF8 protein has been shown to have same midbrain/cerebellum inducing properties than the isthmic tissue grafts (see Fig. 2; Crossley et al., 1996). So far these inducing properties have been demonstrated only for FGF8. Interestingly, only the FGF8b splice format induces ectopic expression in the mouse forebrain of genes normally expressed in the isthmus (Olsen et al., 2006). MHB specific inactivation of $Fgf8$ results to extensive apoptosis in the midbrain-R1 region at E8.5 (Chi et al., 2003). Similarly in $Wnt1$ mutant embryos cells in the midbrain-R1 region die apoptotically around the same stage (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990). In concert with FGF signaling, SHH is thought to regulate the dorsoventral patterning of the midbrain-R1 region (Placzek, 1995; Ye et al., 1998). SHH is expressed ventrally in the floor plate throughout the midbrain-R1 boundary region (see Fig. 1A).

Although there is vast information about the complex genetic interactions controlling the localization of isthmic organizer, the exact factors responsible for its induction are currently unknown. In addition to the early regionalization of the neuroectoderm, it has been suggested that a transient expression of FGF4 in the anterior notochord is needed for the induction of midbrain-R1 specific gene expression (Shamim et al., 1999). The induction of $Fgf8$ expression at the IsO might be due to secreted FGF8 from
the adjacent cardiac mesendodermal cells (Crossley et al., 1996). In addition, other signals from the mesendoderm are required for the induction of the IsO-associated transcription factors such as En1 and En2 (Ang and Rossant, 1993). Recently, the data from the transplantation experiments in chick embryos suggest that convergent WNT and FGF signaling at the gastrula stage is required to generate the cellular properties characteristics for the IsO (Olander et al., 2006; see Fig. 3).

2.3. Neuronal development and regionalization of the midbrain

The dorsal part of the midbrain is formed by two paired rounded structures, the superior and inferior colliculi. The superior colliculus receives input from the retina and the visual cortex and participates in a variety of visual reflexes, particularly the tracking of objects in the contralateral visual field. The inferior colliculus, formed in the caudal midbrain receives both crossed and uncrossed auditory fibres and projects upon the medial geniculate body, the auditory relay nucleus of the thalamus.

The midbrain contains the nuclear complex of the oculomotor nerve (also called III cranial nerve) as well as the trochlear nucleus (also called IV cranial nerve); these cranial nerves innervate muscles that move the eye and control the shape of the lens and the diameter of the pupil. The ventral midbrain region form also two distinct nuclei known as the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) as well as the retrorubral field (RrF), which are the most prominent sources of dopaminergic (DA) neurons in the CNS (German and Manaye, 1993). Indeed, midbrain neurons that use dopamine as a neurotransmitter constitute about 75% of all DA neurons in the adult brain (Wallen and Perlmann, 2003). The location of postmitotic DA neurons in the brain is usually determined by the immunohistological localization of the enzyme tyrosine hydroxylase (TH), which is the rate-limiting enzyme of dopamine synthesis. Historically, the DA neurons in the midbrain of the mouse are placed in three cell groups according to TH immunostaining: nucleus A8 cells in the RrF, nucleus A9 cells in the SNc and nucleus A10 cells in the VTA (Fig. 5).

Figure 5. Midbrain dopaminergic (DA) cell populations during mouse brain development. Signals affecting the early development of DA precursors and patterning of the midbrain-R1 region at E9.5 (A) and the location of TH+ neurons corresponding to A8-A11 groups at E14.5 (B). Di, diencephalon; Ic, inferior colliculi; IsO, isthmic organizer; Mb, midbrain; Sc, superior colliculi; p, prosomere; r, rhombomere; Te, telencephalon. Adapted from reviews by Simon et al. (2003) and Ang (2006).
Cells of the SNc contain the black pigment melanin; these synthesize dopamine and project to cells of the dorsolateral striatum and caudate putamen constituting the nigro-striatal pathway. By contrast, neurons of the VTA and RrF project to the ventral striatum forming a part of the mesolimbic system and establish also additional connections to the prefrontal cortex (mesocortical system). DA neurons of the SNc are involved in the coordination of movements, whereas the VTA and RrF neurons are controlling e.g. mood and rewarding through the mesolimbic and mesocortical systems.

Degeneration of the SNc neurons and their connections to the striatum and frontal cortex is implicated in several CNS disorders, including Parkinson’s disease (Hirsch et al., 1997; Hynes and Rosenthal, 1999). Conversely, over-stimulation of VTA neurons has been linked to schizophrenia and drug addiction (Chao and Nestler, 2004; Sesack and Carr, 2002).

The development of the superior and inferior colliculi and midbrain DA neurons is regulated by the IsO (Andersson et al., 2006a; Brodski et al., 2003; Prakash et al., 2006; Trokovic et al., 2003). The DA progenitors are located rostrally to (or within) the IsO as early as E9.0 in mouse, and they differentiate in this region at a time between E9.0-E14.0 (Hynes and Rosenthal, 1999). The first postmitotic DA neurons can be detected in the ventral midbrain at approximately E10.5 in mouse. The induction of the DA precursors is regulated by both SHH from the floor plate and FGF8 from the IsO (Ye et al., 1998) (see Fig. 5). Addition of SHH and FGF8 on embryonic rat neural tube explants induced DA neuron development in ectopic locations, and blocking of the two signals with antibodies prevented it. Furthermore, in Shh knockouts and conditional Smo (a receptor for SHH signaling) knockouts the amount of midbrain DA progenitors are considerably reduced (Blaess et al., 2006). The induction and maintenance of DA neurons requires also other factors such as TGF-βs (Farkas et al., 2003). Conversely, TGF-α is specifically needed only for the development of DA neurons of the SNc. The inactivation of TGF-α in mice leads to 50% reduction of SNc neurons (Blum, 1998).

A specific marker gene for DA progenitors in the midbrain is retinaldehyde dehydrogenase (also known as Aldh1, Aldh1al or Raldh1), which catalyzes the oxidation of retinaldehyde into retinoic acid. However, the role of Raldh1 in midbrain DA progenitors is not clear (Prakash and Wurst, 2006). The role of WNT signaling in the midbrain DA development has also been established recently. Prakash et al. (2006) showed that Wnt1 is required for the early development of DA neurons through maintenance of Otx2 transcription factor in the midbrain. Recent studies have shown that several other transcription factors are expressed both in DA progenitors and later in immature and mature neurons. Midbrain DA progenitors are expressing a complex set of transcription factors including Otx2, Lmx1a, Lmx1b, En1, En2, Msx1, Msx2, Neurogenin 2 (Ngn2) and Mash1 (Andersson et al., 2006a; Andersson et al., 2006b; Kele et al., 2006; Puelles et al., 2003; Puelles et al., 2004; Simon et al., 2001). These transcription factors as well as Nurr1 and Pitx3 are also important in the later development of DA neurons (Smidt et al., 2004; Smits et al., 2003; Wallen et al., 1999; Zetterstrom et al., 1997). For example, in En1/2 double mutants, which lack the IsO activity, and thus downregulate Fgf8 by E9.0 stage, DA neurons are initially generated (Alberi et al., 2004; Simon et al., 2001; Simon et al.,...
2.4. Neuronal development and regionalization of the hindbrain

During embryonic development cerebellum arises from dorsal R1, adjacent to the fourth ventricle. By contrast, the basal region of R1 will contribute to the development of pons. The cerebellum is essential for fine motor control of movement and the body position, and its dysfunction disrupts balance and impairs control of speech, limb and eye movements. The cerebellum consists of a medial vermis and two laterally located hemispheres. Anatomically, the cerebellum is divided into anterior, posterior and flocculonodular lobes. The developing cerebellum consists mainly of three types of neuronal cells: granule cells in the external germinal layer, Purkinje cells, and neurons of the deep nuclei. The pons functions to relay signals from the cortex to assist in the control of movement where the medulla is joined to the spinal cord and controls unconscious, yet essential, body functions such as breathing, swallowing, blood circulation and muscle tone. Both in mammals and fish, embryological manipulations and gene ablation studies have provided evidence that normal cerebellar development depends on formation and function of the isthmic organizer.

In addition to isthmic signals, such as WNT1 and FGF8, BMP signals from the dorsal R1 and Math1 expression in the rhombic lip characterize the different cell types that form the complex neuronal subtypes in cerebellum. Mice lacking Math1 expression show a complete loss of the cerebellar granule-cell layer and hindbrain mossy-fiber afferent nuclei indicating improper precursor cell differentiation and migration (Ben-Arie et al., 1997). Recent data indicate that BMP signaling pathway regulating Lmx1a transcription factors is required for the development of R1 roof plate and its derivatives (Chizhikov et al., 2006). Thus, in addition to the IsO, the roof plate seems to control both patterning and cellular proliferation in the cerebellar primordium.
The locus coeruleus (LC) is a nucleus in the brain stem responsible for physiological responses to stress and panic. The LC progenitor cells are born in the rostral rhombic lip (Lin et al., 2001), from where they migrate ventrally within the developing neural tube, and take residency near the fourth ventricle. The mature LC is localized in the lateral walls of the fourth ventricle in R1. This nucleus is the main noradrenergic centre of the brain and the main source of noradrenergic innervations in the CNS. The early development of noradrenergic neurons of the LC requires the establishment of IsO and is regulated by a BMP induced signaling cascade that specifies LC progenitors. Initially, BMP signaling pathway activates Mash1 transcription factor in LC progenitors of the adjacent rhombic lip (Guo et al., 1999). Subsequently, Mash1 activates the homeobox genes Phox2a and Phox2b. In the absence of Mash1 (Hirsch et al., 1998), Phox2a (Morin et al., 1997), or Phox2b (Pattyn et al., 2000), the LC neurons do not form. Phox2a and Phox2b also regulate the expression of tyrosine hydroxylase (TH) and dopamine-ß-hydroxylase (DBH), two key enzymes of noradrenergic biosynthesis required for the LC neuronal activity.

The IsO also regulates differentiation of serotonergic neurons (SA) neurons in the anterior hindbrain. These neurons are characterized by the expression of serotonin transmitter (also known as 5-hydroxytryptamine, 5-HT). The serotonergic system plays a key role in modulating behaviors, such as appetite and anxiety and has been implicated in many human disorders of mood and mind. Mammalian SA neurons have been classically grouped into nine cell populations (B1-B9) which are defined by immunohistochemical staining with anti-5-HT. The more rostral SA groups (B1-B4) are located more caudally (reviewed in Hynes and Rosenthal, 1999). The rostral domain (B5-B9) provides innervation mainly to forebrain targets. The regional restriction of 5-HT neuron induction and later specification is believed to result from early signaling events that pattern the MHB region and the more anterior hindbrain. In addition to isthmic FGF8 signals and SHH from the floor plate, FGF4 is needed for SA neuron induction in in vitro cultured R1 explants (Ye et al., 1998), but its role in vivo has not been demonstrated. The homeobox transcription factor Pet1 has been used as a specific marker of developing and adult SA neurons and it is expressed shortly before 5-HT appears in the mouse hindbrain at E11.0. Recently, the differentiation of SA neurons in vertebrates has come under wide examination and has been shown to involve also other homeobox transcription factors. Gain-of-function approaches have implicated that homeobox TFs Pet1 (also known Fev), Lmx1b and Mash1 (also known Ascl1) are required for the specification of all SA neurons whereas Nkx2.2 and zinc finger TF Gata3 are needed for the appearance of caudal SA neurons (Ding et al., 2003; Hendricks et al., 2003; Pattyn et al., 2004).

### 2.4.1. Segmentation and compartmentalization in hindbrain

In mouse, the best studied system for compartmentalization is the early embryonic hindbrain which is characterized by developmental segments called rhombomeres. Rhombomere (R)1 and R2 (metencephalon) give rise to the cerebellum and pons. Boundaries between the rhombomeres coincide with the anterior boundaries of expression of homeobox-containing regulatory genes. Thus, each of
the eight mouse rhombomeres (R1-8) can be characterized by a specific combination of Hox gene expression. However, R1 is devoid of Hox gene expression. In addition, rhombomeres can be identified by the pattern of nerves that emerge from them. Importantly, proliferating cells between the rhombomeres do not mix, as shown by clonal analysis using injected intracellular dye (Fraser et al., 1990), thus proposing that they are compartments. In addition, the compartment boundaries show specific kinetics of cell cycle progression (see section 4.4. below).

The patterns of Hox gene expression imprint a positional code on the cells and contribute to the individual identities of the neural precursors (Guthrie et al., 1991; Guthrie et al., 1992). Segmentation of the vertebrate hindbrain into rhombomeres is essential for the AP patterning of cranial motor nuclei and their associated nerves. For example, a pair of cranial projections originate in single rhombomeric compartment cooperate to form the functional branchiomotor nerve (Lumsden and Keynes, 1989). Segmentation reflects also to the time course of differentiation, where odd-numbered rhombomeres show a delayed maturation compared to the even-numbered rhombomeres. In addition to Hox genes, the vitamin A derivative, retinoic acid (RA), is an early embryonic signal that specifies rhombomeres. RA signaling has an essential role in specifying rhombomere identity by acting upstream of Hox and other transcription factors, particularly in the posterior hindbrain (reviewed by Gavalas and Krumlauf, 2000; Glover et al., 2006; Maden, 2006). These TFs include, for example, Pax2, Meis1, Krox20 and Hoxb1.

Signaling by FGF8 is also crucial in patterning the R1 and establishing the anterior limit of Hox gene expression (Irving and Mason, 2000). There is clear evidence that FGF8 can substitute for IsO activity in inducing R1 character when expressed in hindbrain tissue. The suggestion is that FGF8 is used in restricting the anterior limit of Hox expression by regulating anterior hindbrain character (i.e. R1). Thus both RA and FGF signaling are regulating the Hox expression in the hindbrain. The experiments in frog and fish suggest that FGF and RA signaling work independently to pattern distinct regions along the AP axis and that the Cdx homeobox genes (vertebrate caudal homologs) are responsible for transducing these signals (Isaacs et al., 1998; Shimizu et al., 2006). CDX proteins directly regulate the expression of the posterior Hox genes through direct binding to the cis-regulatory elements of these genes. Therefore the RA and FGF pathways may be integrated at the transcriptional level to control the Hox expression via Cdx genes.

Identities of rhombomeres and their boundaries are also proposed to arise by locally controlled proliferation coupled to cell adhesion changes (Wizenmann and Lumsden, 1997), which results to selective cell mixing. The in vitro affinity experiments by Wizenmann and Lumsden (1997) suggest that distinct cell affinity properties restrict cell mixing between adjacent rhombomeres. Differential expression of cell adhesion molecules is known to be an effective mechanism for cell sorting. Rhombomere-boundary formation through cell sorting is thought to emerge by repulsive Ephrin signaling. For example, repulsive interactions between Eph receptor tyrosine kinase, EphA4, and cells expressing its ephrinB ligands guide the cell sorting that underlies rhombomere-boundary formation (Holder and Klein, 1999; Mellitzer et al., 1999). Moreover, EphA4 expression is showed to promote the intra rhombomeric cell adhesion in parallel with
the EphA4-dependent repulsion between rhombomeres (Cooke et al., 2005).

Apart from Eph-ephrin signaling little is known about the importance of other cell adhesion molecules to the hindbrain segmentation. However, for example the cadherin/catenin system might act as a reciprocal regulator of proliferation and/or cell-cell adhesion in R1 (Trokomovic et al., 2003) as well as in other rhombomeres where disruption of Ca\(^{2+}\)-dependent adhesion abolishes rhombomere-specific cell segregation (Wizenmann and Lumsden, 1997). Thus, differential expression of two cadherin molecules is important for restricting cell movement between adjacent rhombomeres.

3. Intercellular communication in the midbrain-R1 region

3.1. FGF signaling in the midbrain-R1 development

3.1.1. The FGF and FGFR molecules

During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation. Most of the FGFs have a classical amino-terminal signal sequence and are, therefore, efficiently secreted via the endoplasmic reticulum-Golgi secretory pathway. FGF9/16/20 lack an obvious amino-terminal signal peptide, but are nevertheless secreted. Mouse FGFs 11–14 are thought to remain intracellular, and secretion of these FGFs has not yet been reported (Ornitz and Itoh, 2001). Secreted FGFs are molecules which bind their cognate receptors leading to signal transduction in the receiving cells. The FGF receptors (FGFR1-4) belong to the receptor tyrosine kinase family. The extracellular domain of FGFR molecules contains (i) a signal peptide, (ii) three (I-III) immunoglobulin-like (Ig) domains, (iii) an acidic residues of amino acids between Ig-like domains I and II. and (iv) a heparin/heparin sulfate proteoglycans (HS) binding domain (Fig. 6A,B). The Ig-like domains are needed for the FGF ligand binding. In addition, affinity for heparin/HS is essential for effective activation of FGFRs (Ornitz, 2000). FGFRs also contain a single transmembrane domain and a split intracellular tyrosine kinase domain. Upon ligand binding, the receptor dimerizes and undergoes a conformational change which leads to trans-autophosphorylation of FGFR monomers as well as phosphorylation of the FGFR associated molecule FRS2 (Fig. 6A,B).

The use of an alternative exon encoding the carboxyl terminal half of Ig domain III is the major determinant of FGF ligand specificity and FGFR1-3 are present in both IIIb and IIIc isoforms (Fig. 6A,B; Chellaiah et al., 1999; Wang et al., 1995). There are 22 Fgfs in the mouse genome (Zhang et al., 2006) which of Fgf8, Fgf15, Fgf17 and Fgf18 are specifically expressed in the midbrain-R1 region during embryogenesis. FGF8, 17 and 18 are subfamily members which exclusively bind FGFR IIIc splice forms and FGFR4 in vitro (Fig. 6D; Zhang et al., 2006). By contrast, FGF15 subfamily shows relatively weak and unspecific activity for any FGFR1-3c isoform and FGFR4. As mice with Fgfr1IIIb inactivation are phenotypically normal, it has been proposed that the majority of the FGFR1 signaling is carried out by IIIc isoforms (Partanen et al., 1998).
3.1.2. Expression patterns of FGFs and FGFRs in the mouse midbrain-R1

The onset of Fgf8 expression at the midbrain-R1 region is at E8.5 (4-somite stage), slightly later than the Otx2/Gbx2 expression boundary is established (Wurst and Bally-Cuif, 2001). Until at least E12.5 Fgf8 expression domain is detected as a narrow band in the most anterior R1 (Fig. 7). The onset and expression of other Fgfs at the midbrain-R1 region is also well reported. Fgf17 and 18, belonging to the Fgf8 subfamily, are expressed at the midbrain-R1 region already at E8.5 (Xu et al., 2000). Compared to the Fgf8, however, their expression domain is narrower. By E10.5, the level of Fgf17 and Fgf18 expressions is quite similar to Fgf8, but their expression is detected also in the most posterior midbrain (see summarized expression in Fig. 7). Between E10.5 and E12.5 expression of Fgf17 and 18 is detected in the midbrain-R1 region. Expression of Fgf15 in the midbrain-R1 region is initiated at around E9.0 (Gimeno et al., 2002; Gimeno et al., 2003). Transcripts are detected at both sides of the border between midbrain and R1. The negative gap between Fgf15 expression domain in midbrain-R1 region is at least partially overlapping with the Fgf8 expression domain (Gimeno et al., 2003) and it is detectable until E15.5.

The location of Fgfr transcripts at the midbrain-R1 region has been described in several studies (Blak et al., 2005; Ishibashi and McMahon, 2002; Trokovic et al., 2003; expression patterns at E9.5-E10.5 are summarized in Fig. 7 below). Fgfr1 expression is detected broadly in the primitive streak and head folds at E7.5 whereas Fgfr2-3 are more restricted. Between E8.5 and E10.5 Fgfr1 expression is detected broadly in the neural tube and at the border between mid- and hindbrain. Fgfr2 and Fgfr3 are also expressed broadly in the neural tube at these stages.
but a gap in expression at the midbrain-R1 region is detected (Blak et al., 2005; Fig. 7). Interestingly, from at E9.5 onwards Fgfr2 expression in the ventral midbrain is extending posteriorly close to the midbrain-R1 border but do not overlap with the Fgf8 expression (Blak et al., 2005). Fgfr4 expression has not detected at the midbrain-R1 region between E8.5 and E12.5 (Blak et al., 2005).

Although earlier in vitro binding assays (Zhang et al., 2006) have proposed only a weak affinity of FGF8 to FGFR1c (Fig. 6D), the interaction of these molecules during midbrain-R1 development was recently studied using surface plasmon resonance (SPR) assays (Olsen et al., 2006). The SPR analysis showed that the FGF8b splice form is capable to bind to FGFR1c isoform, which is consistent with genetic analyses proposing that FGF8 signals through FGFR1 during midbrain-R1 development (Chi et al., 2003; Trokovic et al., 2003).

3.1.3. FGFRs in the mouse midbrain-R1 development

The inactivation of Fgfr1 or Fgfr2 results in very early lethal phenotypes due to a failure in gastrulation (Arman et al., 1998; Deng et al., 1994). By contrast, Fgfr3 KO mice are viable, but exhibit skeletal and inner ear defects leading to a bone overgrowth and deafness (Colvin et al., 1996; Deng et al., 1996). Because other receptors can compensate for the loss of FGFR3, the role of FGFR3 in neurogenesis was recently studied using the gain-of-function approach (Inglis-Broadgate et al., 2005). In these constitutively active Fgfr3 knockout mice, the regulation of proliferation and apoptosis of cortical progenitors was defective which led to the increased brain size.

Conditional knockout studies of Fgfr1 using En1-Cre and Wnt1-Cre mice have shown its importance in the development of the midbrain-R1 region (Trokovic et al.,

![Figure 7. Summarized gene expression patterns of Fgfs and Fgfrs at E9.5-E11.5.](image-url)
2003). Conditional inactivation of Fgfr1 in the midbrain-R1 region using an Enl-Cre mouse (Kimmel et al., 2000) results in a deletion of the inferior colliculi and vermis of the cerebellum in the dorsal midbrain-R1 region. By contrast, TH-positive neurons were found to be present in newborn and adult conditional Fgfr1 mutants, both in the midbrain DA neurons and in the locus coeruleus (LC) of the R1. In addition, ventral structures such as pontine nuclei as well as mesopontine nuclei were unaffected in conditional Fgfr1 mutants. Although TH-positive cells were present in the LC domain more detailed analysis revealed that the LC appeared to be disorganized in newborn Fgfr1 MHB-specific mutants. Conditional Fgfr1 MHB-specific mutants survived until adulthood, but they showed impaired motor coordination. The phenotype of the conditional Fgfr1 mutants is clearly less severe than the phenotype of the conditional Fgf8 mutants produced with the same Enl-Cre mouse line (Chi et al., 2003). Inactivation of Fgf8 in the MHB led to the extensive cell death in the midbrain-R1 region between E8.5 and E10.0. Consequently, the ectopic apoptosis of MHB cellular progenitors caused the deletion of the entire midbrain, isthmus and cerebellum at E17.5.

The conditional allele of Fgfr2 was also recently generated and the effect of FGFR2 or FGFR3 deficiency on the development of midbrain-R1 region was subsequently studied. Interestingly, the analysis of midbrain-R1 specific Fgfr2 mutant mice and conventional Fgfr3 mutant mice showed that both receptors alone are dispensable for the formation of a normal phenotype and maintenance of the midbrain-R1 region (Blak et al., 2007). Thus, further analyses of FGFR1-3 double and triple mutants are needed to clarify the exact role of FGFR signaling in the midbrain-R1 development.

### 3.1.4. FGFR signal transduction

The phosphorylation events in FGFR tyrosine kinase domain initiate various downstream cascades, such as RAS-mitogen activated protein kinase (RAS-MAPK), phosphatidylinositol 3’ kinase (PI3K/Akt) or phospholipase C gamma 1 (PLCγ1/PKC) pathways (Mohammadi et al., 1991; Mohammadi et al., 1996; Ong et al., 2001). These different pathways are dependent of docking or adaptor proteins with distinct Src-homology 2 (SH2) domains (Ryan et al., 1998). Consequently, the recruitment of different types of SH2 domain proteins triggers a specific network of complex signal transduction cascades.

Upon activation of a FGFR, phosphorylation of tyrosine residues promotes binding of the FRS2 and recruits the GRB2 and SOS adaptor proteins to the complex (Fig. 8). As a result, SOS catalyzes the conversion of RAS from an inactive GDP bound to an active GTP bound form. RAS then triggers the MAPK cascade by recruiting the RAF to the membrane and activates it. The kinase activity of RAS phosphorylates and activates the dual-specificity kinase MEK, which in turn, phosphorylates the ERK. Phosphorylated forms of ERK molecules (pERK1 and 2) can then enter the nucleus where it activates transcription factors, including members of the ETS family of transcription factors.

RAS-MAPK pathway is activated by various ligand-receptor systems, including RTKs and G-protein-coupled receptors. Thus, it is striking that FGF signaling seems to be responsible for nearly all ERK1/2 activity in early frog and fish embryos (Christen and Slack, 1999; Tsang
and Dawid, 2004). In addition, expression of activated pERK has been observed in distinct domains in the mouse neural tube, predominantly in the midbrain-R1 region overlapping with the Fgf8/Fgf17/Fgf18 expression domains (Corson et al., 2003).

PI3K/Akt pathway also plays roles in FGF induced cell proliferation and cell survival. It has been shown that FGF8 induced activation of the PI3K/Akt pathway results to expression of Mkp3 which has a negative feedback action on the RAS-MAPK pathway (Echevarria et al., 2005; Kawakami et al., 2003). During mouse limb development downregulation of Mkp3 by small interfering RNA (siRNA) induced apoptosis in the mesenchyme (Kawakami et al., 2003). In the mouse IsO explants the regulation of Mkp3 expression by FGF seems to be induced by PI3K/Akt pathway (Echevarria et al., 2005). These studies suggest that Mkp3 has a role in mediating the anti-apoptotic signaling through FGF8 induced PI3K/Akt pathway.

The PLCγ1/PKC pathway involves binding of phosphorylated tyrosine 766 residue of FGFR1 (Peters et al., 1992). Upon binding and activation through phosphorylation, PLCγ hydrolyzes phosphatidylinositol-4,5-diphosphate to form two second messengers, inositol-

![Figure 8. FGF-FGFR signal transduction pathway. See text for details. Modified from Jukkola et al. (2006).](image-url)
1,4,5-triphosphate and diacylglycerol. Diacylglycerol is an activator of protein kinase C (PKC), whereas inositol-1,4,5-triphosphate stimulates the release of intracellular Ca^{2+}. In mouse, FGFR1 alleles with a single point mutation at residue 766 replacing tyrosine with phenylalanine (Y766Fl) has been generated (Partanen et al., 1998; Peters et al., 1992). It was shown that this mutant receptor failed to bind PLCγ and the hydrolyzation of phosphatidylinositol as well as Ca^{2+} mobilization was abolished after FGF stimulation (Peters et al., 1992). However, mice homozygous for the Y766Fl allele were viable and fertile and did not show obvious brain or craniofacial defects (Partanen et al., 1998). Analysis of these mouse mutants showed that PLCγ1/PKC pathway through FGFR1 activation is not required for normal pre- or postnatal development or viability.

A set of genes known to be induced in regions of active Fgf8 signaling and thought to either transduce or modulate the FGF signaling pathway is termed as Fgf8 synexpression group. Among the Fgf8 synexpression group members are the transcription factors Pea3 and Erm, and the inhibitors Mkp3, Sef, Spry1 and Spry2 (Fig. 8; see below). In addition to being potential transcriptional targets of FGF signaling, PEA3 and ERM function as transcriptional effectors within cells to transduce FGFR-dependent signals. By contrast, MKP3, SEF and SPRY act within cells as negative feedback inhibitors, modulating and restricting the levels and extent of FGF-FGFR signaling.

In RAS-MAPK signaling cascade, members of the ETS family are the key link between the FGF-FGFR signaling and transcriptional regulation. PEA3 (also known ETV4, Ets variant gene 4) and ERM (also known ETV5, Ets variant gene 5) are thought to be downstream targets of FGF-FGFR signaling (Liu et al., 2003b; Raible and Brand, 2001; Tsang and Dawid, 2004). The action of these transcription factors is hypothesized to promote the FGF signaling (e.g. Gf8 expression) by activating as a positive feedback loop (Tsang and Dawid, 2004). The interactions of ETS transcription factors with the key transcriptional factors such as AP1, SRF and PAX family members further diversify the output of FGF signal transduction, although specific interactions between PEA3 subfamily (PEA3, ERM, ER81) and other key TFs has not been reported (reviewed by Sharrocks, 2001). Members of the PEA3 subfamily have been linked with the specification of neuronal cells (Arber et al., 2000; Roehl and Nusslein-Volhard, 2001). Pea3 or Er81 knockout mice displayed no overt brain phenotypes. In Pea3 -/- mice, however, a male sexual dysfunction was observed, which probably arises owing to neuronal defects (Laing et al., 2000). By contrast, inactivation of Er81 leads to an improper connection between a subset of sensory and motor neurons (Arber et al., 2000). One explanation for these relatively mild phenotypes is that there is redundancy between the members of the PEA3 subfamily. In the midbrain-R1 region, Erm expression is already detected at E8.5 whereas the onset of Pea3 expression is around E9.0 (Chotteau-Lelievre et al., 2001). Between E9.5 and E12.5, Erm and Pea3 expression is seen across the border between midbrain and R1. During development, Er81 expression has not been reported in the midbrain-R1 region.

Members of the Sprouty (SPRY1-4), MAPK phosphatase (MKP) and SEF families are negative modulators of FGF signaling (Fig. 8). SPRY is thought to inhibit FGF signaling by sequestering
GRB2 and preventing its binding to FRS2 (Hanafusa et al., 2002; Minowada et al., 1999), whereas another inhibitory mechanism involves direct interaction of SPRY2 with RAF (Yusoff et al., 2002). Only Spry1 and Spry2 are expressed at the midbrain-hindbrain region between E8.5 and E10.5 (Minowada et al., 1999). The similarity of these expression domains to Fgf8 expression in various regions suggested that they are coordinately regulated. Moreover, overexpression (FGF beads or Spry retrovirus infections) and gene inactivation studies in mouse, chick and zebrafish confirmed that vertebrate SPRYs are functionally conserved and act as FGF-induced feedback inhibitors (Furthauer et al., 2001; Mailleux et al., 2001; Minowada et al., 1999).

More recently, another negative feedback modulator of FGF-FGFR signaling, named MAPK phosphatase-3 (also known MKP3 or DUSP6) has been described in vertebrate limb buds (Eblaghie et al., 2003; Kawakami et al., 2003). MKP3 specifically dephosphorylates and inactivates ERK1/2. In the midbrain-R1 region, Mkp3 is expressed already at E8.5 in a region overlapping with Fgf8 expression domain (Echevarria et al., 2005). Later (E9.5-E10.5), when Fgf8 expression is defined to the most anterior R1, expression of Mkp3 is seen at both sides of the border between midbrain and R1. By siRNA knockdown analysis, Echevarria et al. (2005) showed that in the midbrain-R1 region the negative feedback mechanism of FGFR signaling is modulated by Mkp3 activity. In addition, they proposed that the expression Mkp3 in the midbrain-R1 region is induced by PI3K pathway.

SEF (similar expression to Fgfs; or Il17rd) and FLRT3 are conserved in vertebrates and both encode single-pass transmembrane proteins. The intracellular domain of SEF is required for the interaction with FGFR1 and FGFR2 and subsequent inhibition of FRS2 (Tsang et al., 2002). Studies in zebrafish, mouse and human have shown that SEF inhibits FGF-induced activation of RAS-MAPK pathway, and mouse SEF also inhibits FGF-induced activation of protein kinase B (pKB/Akt), a key protein in the PI3K pathway (Kovalenko et al., 2003; Yang et al., 2003). There are at least two alternatively spliced isoforms of SEF in humans which are not reported in mice. SEF-a is an isoform that can interact with the receptors whereas another isoform, SEF-b, lacks the signal peptide and therefore it is cytoplasmic and suppresses RAS-MAPK pathway downstream of MEK (Preger et al., 2004). Between E8.5 and E12.5 Sef expression at the midbrain-R1 regions overlaps with the Fgf8, although its expression domain is broader (Lin et al., 2002).

FLRT3 together with FLRT1 and FLRT2, compose a novel gene family which were first isolated in a screen for extracellular matrix proteins expressed in muscle cells (Lacy et al., 1999). The three genes encode a highly conserved family of glycosylated proteins (FLRTs) containing a fibronectin III domain and leucine rich repeats (LRR) domain. Extracellular LRR repeats are commonly involved in protein-protein interactions. In particular, LRRs are common motifs in the extracellular region of transmembrane proteins and also in secreted proteins that are involved in ligand–receptor interactions or in cell adhesion. The former was supported by in vitro protein-protein interaction studies, both in Xenopus laevis (Bottcher et al., 2004) and in mouse (Haines et al., 2006), suggesting that members of the FLRT family can directly interact
with the different FGFRs. In addition, overexpression studies with *Xenopus Flrt3* have suggested that FLRTs may promote FGFR signaling in vertebrates (Bottcher et al., 2004). In *Xenopus*, the role of FLRT3 in promoting FGFR signaling was further hypothesized by the overlapping *Flrt3* and *Fgf8* expression patterns, particularly in the midbrain-R1 region (Bottcher et al., 2004). Recent data suggest, that FLRTs may also mediate homophilic cell adhesion and cell sorting (Haines et al., 2006; Karaulanov et al., 2006). Thus, FLRTs may represent yet another family of cell-surface proteins that link cellular adhesion and signaling through transmembrane receptor.

**CANOPY1** (CNPY1) is the latest member of the *Fgf8* synexpression group. It was found in a screen for zebrafish genes expressed at the isthmus in response to signaling between the midbrain and the isthmus (Hirate and Okamoto, 2006). *Cnpy1* was specifically expressed in the zebrafish midbrain-R1 region. Sequence analysis revealed that *Cnpy1* is one of four structurally related proteins (CNPY1-4) conserved from mouse to human. Among Canopy family proteins, only *Cnpy1* is specifically expressed in the midbrain-R1 region of zebrafish embryos. Human CNPY1 is localized in the endoplasmic reticulum and it may interact with the extracellular domain of the FGFR1. CNPY1 has structural similarity to saposins, proteins that bind sphingolipids, and it was speculated that CNPY1 could influence association of the receptor with lipid rafts. It was also shown that implantation of FGF8b-soaked beads in the midbrain of zebrafish embryos increased expression of *Cnpy1* (Hirate and Okamoto, 2006). In addition, experiments in which expression of CNPY1 protein was blocked with antisense morpholino oligonucleotides showed that *Cnpy1* was essential for normal development of the zebrafish tectum and cerebellum.

**Constant proliferation of neuroepithelial cells** in the midbrain-R1 region causes progressive growth of caudal midbrain and rostral hindbrain areas, respectively. However, the expression of *Wnt1* and *Fgf8* is constantly restricted to a band of only a few cell-diameters wide (e.g. app. 10 cell-layers of *Fgf8* expressing cells in E10.5) at both sides of the border between midbrain and R1. Interestingly, the expression of MAPK pathway inhibitors such as *Mkp3* as well as FGFR signal transduction activators such as *Cnpy1* is detected in a broader midbrain-R1 region compared to *Fgf8*. It is thought that the extracellular spreading of FGF8 in a tissue is due to a diffusion-based mechanism controlled by an active endocytosis of the target cells (Scholpp and Brand, 2004). In these analyses, spreading of FGF8 protein labeled *in vitro* with Cy3 fluorophore from a local source was monitored to determine how the molecule spreads through a target tissue. Reducing the rate of endocytosis led to an extracellular accumulation of FGF8 and resulted in broader domains of FGF-target gene expression, suggesting an increased spreading of FGF8. Thus, different levels of endocytosis allow different extent of spreading of FGF8 protein through the target tissue, leading to differential target gene response. However, the endogenous graded activity of FGF8 or other FGF proteins has not been directly visualized around the IsO.

### 3.2. WNT signaling in the midbrain-R1 development

In addition to FGF-FGFR signaling, WNT signaling has an important role in early embryonic patterning through the
regulation of differentiation, cell death, tissue polarity and migration. WNTs are secreted glycoproteins which can influence tissue organization and growth by functioning locally or on adjacent cells. Moreover, they can also act at a distance by generating a gradient across a several cell layers. Also like FGFs, WNT proteins act on cell surface by binding to receptors called frizzleds (FZ) which are members of the seven transmembrane domain cell surface receptors (Fig. 9). Interactions with complexes between FZ and the LDL receptor-related protein (LRP) molecules have been shown to mediate the signaling activities of WNTs. The WNT-FZ-LRP receptor complex activates dishevelled (DVL) in the cytoplasm. Downstream of DVL, the WNT pathway diverges into at least three branches: the canonical or WNT/β-catenin pathway, the planar cell polarity (PCP) pathway and the WNT/calcium pathway. The patterning of the midbrain-R1 region by WNTs is probably through canonical pathway, as specific inactivation of β-catenin in the MHB mimics the Wnt1−/− phenotype (Brault et al., 2001). In the WNT/β-catenin pathway the activation of DVL leads to the inactivation of glycogen synthase kinase 3β (GSK3β) in the axin-based complex. This in turn stabilizes the β-catenin and allows its relocation to the nucleus where it promotes the transcription by interacting with members of the TCF family of HMG box containing proteins. LRPs are especially needed for the canonical pathway (Pinson et al., 2000).

The most important WNT molecule for midbrain-R1 development is found to be WNT1 (McMahon and Bradley, 1990; Thomas et al., 1991). Although WNT1 does not affect the induction of Fgf8 it is required for the maintenance of FGF8

**Figure 9. Canonical WNT pathway.**

Low density lipoprotein (LDL) receptor-related proteins (LRP5 and 6 in mouse) are required signaling co-receptors for the canonical Wnt pathway (Pinson et al. 2000). Secreted WNT signaling antagonists such as Dickkopf 1 (DKK1) can interact with LRP5/6 proteins. Other secreted antagonists such as Cerberus or Frizzled-related proteins (e.g. FRZB1 or SFRP2) can bind WNTs directly thus presumably inhibiting WNT-FZ interactions. Activation of DVL induces the disassembly of a complex consisting of AXIN, adenomatosis polyposis coli (APC), GSK3β and β-catenin. In the absence of WNT activity, the GSK3β of this complex phosphorylates β-catenin, thereby triggering its degradation. Modified from Bouwmeester et al. (1996), Houart et al. (2002) and Leyns et al. (1997).
expression, possibly through EN1. At E8.0 (0 somite stage), after the onset of Otx2 and Gbx2 expression Wnt1 is detected in a broad domain. Initially, Wnt1 expression overlaps partially with the Gbx2 domain, but becomes restricted to the Otx2-positive domain in the midbrain at 6 somite stage (Bally-Cuif et al., 1995). Subsequently, Wnt1 expression is restricted to a narrow band and its expression at the midbrain-R1 region is maintained at least until E14.5.

A number of β-catenin-TCF target genes have been identified in diverse biological systems and it is becoming clear that the majority of WNT target genes are tissue specific (Ciani and Salinas, 2005). Similarly to FGF-FGFR signal transduction, numerous WNT target genes appear to be positive and negative regulators of the pathway. These include positive regulators, such as FZ receptors, LRPs, and TCF/LEF and negative regulators, such as AXIN2 and SFRPs, which all are activated by the β-catenin-TCF transcription machineries. The best candidates for general downstream targets of β-catenin-TCF are AXIN2 and SP5 which are expressed in various tissues in vertebrates (Jho et al., 2002; Weidinger et al., 2005). In frog, En2 is a direct target of WNTs at the midbrain-R1 boundary region, but this has not been demonstrated in mouse (McGrew et al., 1999).

The role of other WNTs in the development of midbrain-R1 region has not been established in the mouse. In zebrafish, knockdown studies have shown that Wnt1 and Wnt10b provide partially redundant functions in the development of midbrain-R1 region (Buckles et al., 2004; Lekven et al., 2003). There are also indications that zebrafish Wnt8 is required for the early positioning of the IsO (Rhinne et al., 2005).

4. Cell proliferation in the developing brain

In order to generate appropriate number of neurons and glia in the correct spatial and temporal patterns, it is critical to regulate the number of progenitor cells. In addition the timing of cell differentiation (i.e. the exit from the cell cycle) is crucial for the size, shape and histogenesis of many tissues, including the brain.

The initial proliferation of neural progenitors in developing CNS is limited to the ventricular zone (VZ) that lines the lumen of the neural tube. The majority of neural progenitors give rise to postmitotic neurons by asymmetric divisions that migrate into more apical zones to form marginal layers. Although mitotic nuclei dynamically change their vertical location within the VZ coincidentally with the progression of the cell cycle, neural progenitors maintain a pseudo-stratified columnar epithelium by linking with each other by adherens junctions.

4.1. Interkinetic nuclear migration

The adult brain is composed of neurons and glia, defined by cellular morphology, cell density and the formation of distinct neurites. During development, different types of cells are generated in the pseudostratified epithelium that forms the ventricular zone (VZ), a layer of dividing progenitor cells at the ventricular surfaces of the developing neural tube (Bayer et al., 1991; Sauer 1935 in Developmental Neurobiology, Jacobson & Rao Eds. 2005). As progenitor cells progress through the cell cycle their nuclei undergo dynamic intracellular migration, called interkinetic nuclear migration (Sauer 1935). During this process (Fig. 10), nuclei move away from the apical surface
(ventricular side) during G1, reside in the basal half of the VZ (pial side) during S phase and return apically in G2 phase so that mitosis occurs at the VZ (Bayer and Altman, 1991; Sauer 1935). After mitosis, a portion of the dividing cells exit the cell cycle (post mitotic or quiescent/G0 phase), whereas a complementary portion re-enters S phase and sustains the proliferative pool. Thus after a cell division, a daughter cell can either repeat or exit the cell cycle. The degree to which mitotic cleavage and interkinetic nuclear migration prevails provide a mechanism for the balance between cell cycling and differentiation. It has been hypothesized that the self-renewing progenitor cells are dividing symmetrically (Fig. 10A), in a plane roughly parallel to the ventricular surface to produce two identical daughter cells thus expanding the amount of stem cells or progenitors (proliferative pool). By contrast, the progress of neurogenesis (i.e. differentiation), occurs asymmetrically (Fig. 10B) in a plane roughly perpendicular to the ventricular surface. These self-renewing divisions generate a daughter stem-cell/progenitor cell.

Figure 10. Symmetrical versus asymmetrical cell division. The cells of the neuroepithelium widen the width of the neural tube by dividing symmetrically in the plane of the ventricular surface (yellow arrow in A). Divisions perpendicular to the plane of the epithelium are thought to be asymmetric (red arrow in B) and generate a neural cell or quiescent cell (G0 phase). Modified from the text book: Developmental Neurobiology, Jacobson & Rao Eds. 2005.
and a more differentiated cell type such as a non-stem-cell progenitor or a neuron (Gotz and Hutten, 2005). As neuronal production proceeds during the development, asymmetrical division starts to predominate (after E12.5 in mouse). However, toward the end of the neurogenesis, non-stem-cell progenitors revert back to symmetrical divisions which start to predominate, but produce two identical terminally differentiated neurons (green cell in Fig 10A).

4.2. Cell cycle regulators

4.2.1. Cyclins, Cdks and their inhibitors

The genes that control the number of cell divisions in the vertebrate CNS are beginning to be understood. Cyclins are a group of proteins that show ‘cyclic’ changes in their expression levels that correlate with specific phases of the cell cycle (Fig. 11A). Cyclins bind their catalytic partners, cyclin-dependent kinases (CDKs), and it is now clear that CDKs, either directly or indirectly control the major cell-cycle transitions and phases in all eukaryotic organisms. Different cyclin/CDK interactions are required at different phases of the cell cycle. The association of cyclins with CDKs results to phosphorylation of retinoblastoma susceptibility protein (pRb) and progression to the next phase of the cell cycle through transcriptional activation of target genes (Deng et al., 1995; Shuttman et al., 1999). In mammalian cells, there are two classes of CDKs that function at the G1/S phase transition (Sherr, 1994). CDK4 and its close relative CDK6 are driven by three D-type cyclins (CyclinD1-D3). By contrast, CyclinE activates specifically CDK2. CyclinE accumulates very close to the G1/S phase transition and has a role in phosphorylating pRb (Ciemerych and Sicinski, 2005). CyclinE expression is more periodic than that of D-type cyclins (Roberts and Sherr, 2003). CyclinA also activates CDK2 that plays role in the S phase progression. B-type cyclins (CyclinB1-B3 in mouse) as well as

Figure 11. The phases of cell cycle and cell cycle gene expression. (A) The diagram shows the period in the cell cycle when each of the cyclins is expressed. Cdns are expressed more or less throughout the cell cycle, but their activation (phosphorylation) is phase specific. A cell proliferation marker protein, Ki67, distributes to the chromosome periphery during mitosis and nucleolar heterochromatin in the interphase. (B) Regulation of CyclinD-CDK4/6 complex. The association of CyclinDs with CDKs depends, for example, on the Cip/Kip family of CKIs. It is also negatively regulated by INK4 inhibitors. Once assembled CyclinD-CDK4/6 complexes are subjected to inhibition by Cip/kip inhibitors such as p21 and p27. Adapted from Roberts (1999).
CyclinA together with their kinase partner CDK1 (also called CDC2), represent the important controllers of the G2/M phase progression (Ciemerych and Sicinski, 2005).

CyclinD1 (also called CYCD1 or CCND1 in MGI) together with associated kinases (CDK4 and CDK6) phosphorylate the substrate proteins (e.g. retinoblastoma protein, Rb) that lead to release of E2F transcription factor. For example pRB phosphorylation by CyclinD1/CDK4 complex is necessary for progression from the G1 phase to S phase (DNA synthesis). In contrast to other cyclins, which are induced periodically during cell cycle progression, the expression of D-type cyclins is controlled largely by the extracellular environment (reviewed in Roberts, 1999).

Regulation of cyclin/CDKs complexes occurs at multiple levels, including assembly of cyclin and CDK subunits, phosphorylation and dephosphorylation events and association of cyclin/CDK complexes by inhibitory proteins (Fig. 11B). These regulators include CAKS (CDK activating kinases) and phosphatases, such as Drosophila and vertebrate orthologues string and CDC25, respectively (Kakizuka et al., 1992). In mouse, the activity of one of three CDC25 tyrosine phosphatases on CDK1 results in full activation of CyclinB-CDK1 complex and cell’s entry into mitosis (Galaktionov and Beach, 1991). In addition, there are number of CDK inhibitors (cyclin-dependent kinases inhibitors, CKIs), which bind to CDKs and block their catalytic activity (Sherr and Roberts, 1999). They are grouped to two classes called the Cip/Kip family and the INK4 family. The Cip/Kip family is composed of three members p21, p27 and p57 which can bind to and inhibit all known CDKs (Deng et al., 1995; Kukoski et al., 2003; Shtutman et al., 1999). The INK4 family is composed of four members: p15, p16, p18 and p19 and they specifically inhibit the CDK4/CDK6 activity (Sherr and Roberts, 1999).

4.2.2. Cyclins and Cdk in neuronal development

During mouse development D-type cyclins are expressed in a tissue-specific, but greatly overlapping manner (Ciemerych et al., 2002; Wianny et al., 1998). At E8.5, CyclinD1 is expressed broadly in the midbrain-R1 region whereas CyclinD2 expression is absent from rhombomeres 1 and 2. Later between E9.5 and E11.5 CyclinD1 and CyclinD2 expression is detected strongly at the midbrain-hindbrain region in an overlapping pattern. Mice lacking the individual cyclins (CyclinD1, D2 or D3) have been generated and the observed defects in neurogenesis were relatively mild. In summary, CyclinD1 deficient mice showed reduced body size and neurological abnormalities caused by an unknown mechanism (Fantl et al., 1995; Sicinski et al., 1995). By contrast, CyclinD2 KOs exhibited cerebellar defects which were showed to be due to a decreased proliferation of granule cell precursors (Huard et al., 1999). In addition, the adult CyclinD2 knockout mice lack newly born neurons in the dentate gyrus of the hippocampus and in the olfactory bulb (Kowalczyk et al., 2004). Neurological defects were not reported in CyclinD3 knockouts (Sicinska et al., 2003). Mice lacking the combinations of the D-type cyclins (double and triple KOs) represented the sum of the phenotypes observed in the individual CyclinD mutants (Kozar et al., 2004). Thus, these analyses suggested that the proliferation of several neuronal cell types could take place in a CyclinD-independent fashion (Kozar et al., 2004).
The role of other type of cyclins such as CyclinA or CyclinB in neuronal development has not been reported. These cyclins together with their kinase partner CDK1, represents the major regulators of the G2/M phase progression. Both CyclinA2 and CyclinB1 knockout mice showed an early embryonic lethality (Brandeis et al., 1998; Murphy et al., 1997) whereas CyclinA1 or CyclinB2 mutants were viable, but no overt CNS phenotypes were observed (Brandeis et al., 1998; Liu et al., 1998). The expression of A- and B-type of cyclins during the early brain development has not been reported.

In conventional mutant mice deficient for the Rb protein, progenitor proliferation in the CNS is profoundly deregulated, resulting in excess dividing cells localized to normally post-mitotic regions (Clarke et al., 1992). These mice lacking Rb die around E15.5 due to a severe anemia. More recently, Wu et al. (2003) found that the failure of the placental development caused the early lethality. To circumvent the placental abnormalities they produced rescued Rb-deficient mice with a wild-type placentas using tetraploid complementation technique. Interestingly, no developmental defects in the CNS were observed in these rescued mice (de Bruin et al., 2003; Wu et al., 2003).

### 4.2.3. Cdk inhibitors in neuronal development

Critical negative regulators of the cyclin/CDKs p21 are p27 are expressed in the developing and adult nervous system in the differentiating neurons shortly after they have withdrawn from the mitotic cycle (Parker et al., 1995). The development of neurons in the CNS requires that progenitor cells leave the cell cycle and activate specific programs of differentiation and migration. Genetic studies have identified some of the molecules controlling these cellular events, but how the different aspects of neurogenesis are integrated into a coherent developmental program remains unclear. One possible mechanism implicates multifunctional proteins that regulate both cell cycle exit and cell differentiation. The expression of p21 during mouse development correlates with terminally differentiating cells such as olfactory neurons (Deng et al., 1995; Parker et al., 1995). However, the fact that p21 knockout mice appears to have normal brain development, indicates that any essential role for p21 in terminal differentiation must be redundant (Deng et al., 1995). In the other hand, p27 deletion in mice causes a phenotype opposite of that of the CyclinD1/D2 double knockout, which shows reduced body size and hypoplastic cerebella. In p27 KO animals, the gene inactivation causes an overproduction of neuronal cells (Kiyokawa et al., 1996; Nguyen et al., 2006). p27 has recently been shown to function beyond cell cycle regulation and promote both neuronal migration and differentiation of newborn cortical neurons, through distinct and separable mechanisms. A report by Nguyen et al. (2006) shows that cytoplasmic p27 protein promotes neuronal migration by direct blocking of the small GTPase RhoA and its downstream pathway. Morover, p27 can act as a Neurogenin 2 (NGN2) stabilizer and promotes neuronal differentiation.

Little is known about the regulation of cell cycle inhibitors by transcription factors. In vitro overexpression of basic HLH genes such as NeuroD in the P19 or HeLa cell lines can induce p27 or p21, respectively (Farah et al., 2000; Mutoh et al., 1998). The p21 promoter sequence contains multiple bHLH activator-binding sites (E-boxes) which have been shown to be functional in the up regulation
Elevated $p21^{\text{expression}}$ and ectopic mitosis are observed also in the enteric epithelium of NeuroD-knockout mice (Mutoh et al., 1998). In addition, the basic helix-loop-helix genes $Hes1$ which is an essential effector for Notch signaling can repress expression of the $p21$ in vitro (Castella et al., 2000; Kabos et al., 2002).

4.3. Mechanisms controlling the cell polarity and asymmetric cleavage

The mechanisms that regulate the interkinetic nuclear migration and the transition from predominantly symmetrical to predominantly asymmetrical divisions are poorly understood. Apico-basal polarity is an essential characteristic for interkinetic nuclear migration. Recently, the role of small GTPase CDC42 in the maintenance of apico-basal polarity and self-renewal was showed in the cerebral cortex (Cappello et al., 2006). Conditional inactivation of $Cdc42$ at different developmental stages demonstrated that $Cdc42$ is needed for the apically directed interkinetic nuclear migration. In addition to polarity, other cellular mechanisms such as cell cycle length, orientation of mitosis and basement membrane composition are thought to regulate the decisions between progenitor cell proliferation and differentiation. Clonal analysis on oligodendrocyte progenitor cells suggested that an intrinsic clock operates within each cell to assist control when the cell stops dividing and differentiates (Temple and Raff, 1986). Thus, a similar “cellular” clock, which can be influenced by extrinsic signals, may act to control the balance between the cell cycle progression and differentiation also in neural progenitors. For example, at the start of cortical neurogenesis $Pax6$ inactivation resulted in the increased cell cycle length and the premature expression of neural-specific markers, such as TUJ1 (Estivill-Torrus et al., 2002). Reduction of the cell cycle length by decreasing G1 phase increases the likelihood of cell cycle re-entry and might expand the pool of neuronal progenitor cells. For example, the transition of neuroepithelial to radial glial cells and their progression from proliferative to differentiating divisions during embryonic development is associated with an increase in the length of their cell cycle (Cappello et al., 2006). Remarkably, this increase in cell-cycle length is predominantly due to a lengthening of the G1 phase.

In addition to the apico-basal polarity, a plane parallel to the plane of neuroepithelium defines planar polarity, which plays a role in the development of pattern across a field of cells. The planar polarity and signals may also contribute to cell cycle progress in adjacent brain compartments (Guthrie et al., 1991).

4.4. Cellular proliferation and regulation in the rhombomeric boundaries

It is suggested that the highest level of proliferation is detected at rhombomeres whereas the inter rhombomeric boundaries show reduced rates of proliferation/cell cycle progression (Guthrie et al., 1991). Guthrie et al. (1991) showed that at rhombomere boundaries DNA synthesis (S-phase) occurs closer to the ventricular surface compared to cell within rhombomeres where S-phase cells were detected more basally. The molecular mechanisms and regulators responsible for these changes in nuclear migration patterns in the rhombomeric boundaries are currently unknown. Later in development, the proliferative cells of ventricular zone are restricting the cell mixing between rhombomeres after evident hindbrain
segmentation disappears (Wingate and Lumsden, 1996).

Cadherins mediate cell adhesion and play a fundamental role in normal development. In the neuroepithelium, they participate in the maintenance of proper cell-cell contacts. Cadherins are associated with the actin cytoskeleton through β- and α-catenins to maintain adherens junctions between cells (Nelson and Nusse, 2004). The canonical WNT pathway acts also at the level of transcription, wherein the WNT signal stabilizes and increases β-catenin in the cytoplasm and nuclei. β-catenin regulates the expression of numerous target genes, including CyclinD1 that progresses through the G1-to-S-phase cell-cycle transition (Shtutman et al., 1999). Because cadherins sequester β-catenin into the adhesion complex on the cell surface, it is thought to have dual regulatory roles there, acting positively on cell adhesiveness and negatively on the WNT canonical pathway, which can reduce the progression of the cell cycle (Nelson and Nusse, 2004).

In rhombomeres, CyclinD1 and CyclinD2 show distinct segments specific expression patterns, which are temporally regulated along the AP axis (Wianney et al., 1998). The rhombomere specific expression of CyclinD1/2 could be the one mechanism to regulate the length of the G1 phase in rhombomeres. Interestingly, restricted expression of CyclinD1 and CyclinD2 are detected before the formation of inter-rhombomeric boundaries (3-5 somite stage).

Several Wnt genes are expressed in developing hindbrain. For example, Wnt1 and Wnt3a are expressed in the zebrafish hindbrain. In addition, the zebrafish Wnt genes (Wnt1, Wnt3a, Wnt8b, and Wnt10b) show elevated expression at rhombomeric boundaries (Riley et al., 2004). Consequently, partial inactivation of WNT signaling by knockdown of zebrafish Wnt1 or Tcf3b results to deregulation of boundary regions (Riley et al., 2004) and ectopic expression of boundary cell markers, Rfng (radical fringe) and Foxb1.2, in non-boundary regions of the hindbrain (Amoyel et al., 2005). In the latter, authors showed that the Wnt1-positive cells expressed in the rhombomeric boundary and roof plate contributed to upregulation of proneural and Delta gene expression. As Delta cell-autonomously blocks Notch activation, this pathway mediates lateral inhibition that prevents spreading of the Wnt1 boundary expression domain. Thus, boundary cells play a key role in the regulation of cell differentiation in the zebrafish hindbrain (Amoyel et al., 2005). These data suggest that WNT and Notch-Delta signaling regulate neurogenesis in non-boundary regions, which in turn blocks ectopic boundary marker expression. Similar feedback mechanisms operate in the Drosophila wing disc and vertebrate limb bud, suggesting adaptation of a conserved signaling module that spatially organizes cells in complex organ systems. In the mouse, however, only the midbrain and cerebellum is deleted in mice mutant for Wnt1 (McMahon and Bradley, 1990; Thomas et al., 1991) suggesting redundancy between WNT signals.

These results obtained from the rhombomeric boundaries contribute to our understanding of how the cell cycle control can be linked to the patterning programs to influence the balance between proliferation and neuronal differentiation in discrete progenitor domains. An analogous situation to rhombomeric boundaries occurs in the IsO in which a signaling center expressing Fgf8 and Wnt1 in the adjacent brain regions. Thus, the cellular mechanisms characteristic for the rhombomeric cell cycle regulation might be similar in the IsO.
AIMS OF THE STUDY

The aim of this study was to characterize the molecular and cellular mechanisms responsible for the midbrain and rhombomere 1 patterning defects observed in the conditional Fgfr1 mutant mice.

The aims were:

I. To identify FGFR1 and/or IsO target genes (I).
II. To characterize expression of one of these, Drapc1 (II).
III. To study the FGF-regulated neurogenesis in the midbrain-R1 region (I).
IV. The aim was also to study the cell cycle regulation at the midbrain-hindbrain boundary (III, IV).
MATERIALS AND METHODS

Methods, mouse lines, probes and antibodies used in this study are listed in tables 1-4 and protocols and sources are referenced therein.

Table 1. Methods used in studies I-IV.

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference/Source</th>
<th>Study number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU incorporation and immunohistochemical detection</td>
<td>Cell proliferation kit (Amersham-Pharmacia)</td>
<td>III, IV</td>
</tr>
<tr>
<td></td>
<td>- see table 4</td>
<td>I, III-IV</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microarray analysis</td>
<td>Affymetrix GeneChip mouse U74A v2 arrays</td>
<td>I</td>
</tr>
<tr>
<td>- Total-RNA extraction</td>
<td>Trizol reagent (Invitrogen)</td>
<td>I</td>
</tr>
<tr>
<td>- Northern blot analysis of isolated total-RNA</td>
<td>(Frilander and Steitz, 1999)</td>
<td>I</td>
</tr>
<tr>
<td>- cRNA labeling</td>
<td>BioArray High Yield RNA Transcript Labeling kit (Enzo)</td>
<td>I</td>
</tr>
<tr>
<td>- Data analysis</td>
<td>MAS 5.0 / GeneSpring software</td>
<td>I</td>
</tr>
<tr>
<td>Radioactive section in situ analysis</td>
<td>Wilkinson and Green 1990</td>
<td>I-IV</td>
</tr>
<tr>
<td>PCR for genotyping</td>
<td>(Trokovic et al., 2003)</td>
<td>I-IV</td>
</tr>
<tr>
<td>TUNEL assay</td>
<td>In situ cell death detection kit (Roche) - see table 4</td>
<td>III</td>
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<tr>
<td>Whole-mount in situ hybridization analysis</td>
<td>(Henrique et al., 1995)</td>
<td>I-IV</td>
</tr>
<tr>
<td>ß-galactosidase staining</td>
<td>(Lobe et al., 1999)</td>
<td>IV</td>
</tr>
<tr>
<td>NADPH-diaphorase histochemistry</td>
<td></td>
<td>IV</td>
</tr>
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Table 2. Mouse lines used in this study. All animal procedures were performed in accordance with the national guidelines and instructions and the experiments were approved by the committee of experimental animal research of the University of Helsinki.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Reference</th>
<th>Study number</th>
</tr>
</thead>
<tbody>
<tr>
<td>En1-Cre</td>
<td>(Kimmel et al., 2000)</td>
<td>I-III</td>
</tr>
<tr>
<td>Fgfr1&lt;sup&gt;lox&lt;/sup&gt;</td>
<td>(Trokovic et al., 2003)</td>
<td>I-III</td>
</tr>
<tr>
<td>En1&lt;sup&gt;+/Wnt1&lt;/sup&gt;</td>
<td>(Panhuysen et al., 2004)</td>
<td>III</td>
</tr>
<tr>
<td>FST KO</td>
<td>(Matzuk et al., 1995)</td>
<td>I</td>
</tr>
<tr>
<td>Trh KO</td>
<td>(Yamada et al., 1997)</td>
<td>not published</td>
</tr>
<tr>
<td>MHB-Cre</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>R26R</td>
<td>(Soriano, 1999)</td>
<td>IV</td>
</tr>
</tbody>
</table>
Table 3. Probes used for *in situ* hybridization.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Annotation / Reference</th>
<th>Study number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Mouse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aldh1</em></td>
<td>(Hermanson et al., 2003)</td>
<td>I</td>
</tr>
<tr>
<td><em>CyclinD1</em></td>
<td>IMAGE 3155470</td>
<td>III</td>
</tr>
<tr>
<td><em>Fgfr1</em></td>
<td>(Trokoivc et al., 2003)</td>
<td>III</td>
</tr>
<tr>
<td><em>Fgfr2</em></td>
<td>(Trokoivc et al., 2003)</td>
<td>III</td>
</tr>
<tr>
<td><em>Fgfr3</em></td>
<td>(Trokoivc et al., 2003)</td>
<td>III</td>
</tr>
<tr>
<td><em>Gata3</em></td>
<td>(Lillevali et al., 2004)</td>
<td>I</td>
</tr>
<tr>
<td><em>Hes3</em></td>
<td>clone UI-R-BO1-ajt-c-02-0-UI.r1</td>
<td>I</td>
</tr>
<tr>
<td><em>Lmx1b</em></td>
<td>a gift from H. Simon</td>
<td>I</td>
</tr>
<tr>
<td><em>Mash1</em></td>
<td>IMAGE 6415061</td>
<td>I</td>
</tr>
<tr>
<td><em>Ngn2</em></td>
<td>IMAGE 2922473</td>
<td>I</td>
</tr>
<tr>
<td><em>Nurr1</em></td>
<td>(Wallen et al., 1999)</td>
<td>I</td>
</tr>
<tr>
<td><em>Otx2</em></td>
<td>(Acampora et al., 1997)</td>
<td>I,III,IV</td>
</tr>
<tr>
<td><em>Pb-cadherin</em></td>
<td>(Trokoivc et al., 2003)</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>Phox2a</em></td>
<td>IMAGE 534970</td>
<td>I</td>
</tr>
<tr>
<td><em>Pitx3</em></td>
<td>IMAGE 482871</td>
<td>I</td>
</tr>
<tr>
<td><em>p21</em></td>
<td>A gift from Bert Vogelstein</td>
<td>III,IV</td>
</tr>
<tr>
<td><em>Sert (Slc6a4)</em></td>
<td>A gift from Wolfgang Wurst</td>
<td>I</td>
</tr>
<tr>
<td><em>VaChT</em></td>
<td>(Trokoivc et al., 2003)</td>
<td>I</td>
</tr>
<tr>
<td><em>Wnt1</em></td>
<td>(Trokoivc et al., 2003)</td>
<td>I</td>
</tr>
<tr>
<td><strong>B) Chicken ESTs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Drapc1 (or Pogo1)</em></td>
<td>ChEST ID: C000369</td>
<td>unpublished</td>
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<td></td>
<td>ChEST ID: C0003888</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>ChEST ID: C0001388</td>
<td>&quot;</td>
</tr>
<tr>
<td><strong>C) Zebrafish</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cnpyl</em></td>
<td>IMAGE 7068815</td>
<td>I</td>
</tr>
</tbody>
</table>

Other probes and their references are listed in the results section (Tables 5 and 6).

*B) Chicken cDNAs ordered from ARK-Genomics (Boardman et al. 2002).
### Materials and Methods

#### Table 4. Antibodies used in immunohistochemical analyses.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description, dilution &amp; supplier</th>
<th>Study number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-β-galactosidase</td>
<td>rabbit polyclonal (1:2000, Cappel)</td>
<td>IV</td>
</tr>
<tr>
<td>anti-BrdU</td>
<td>mouse monoclonal (1:500, Amersham)</td>
<td>III, IV</td>
</tr>
<tr>
<td>anti-BrdU</td>
<td>sheep monoclonal (1:800, Abcam)</td>
<td>IV</td>
</tr>
<tr>
<td>anti-KI67</td>
<td>rabbit polyclonal (1:500, Neomarkers)</td>
<td>IV</td>
</tr>
<tr>
<td>anti-p21</td>
<td>mouse monoclonal (1:100, BD Biosciences)</td>
<td>IV</td>
</tr>
<tr>
<td>anti-phosphohistone-H3 (PH3)</td>
<td>rabbit monoclonal (1:800, Upstate)</td>
<td>III</td>
</tr>
<tr>
<td>anti-Tuj1 (or III β-Tubulin)</td>
<td>rabbit monoclonal (1:300, Covance)</td>
<td>I</td>
</tr>
<tr>
<td>anti-tyrosine hydroxylase (TH)</td>
<td>rabbit monoclonal (1:500, Chemicon)</td>
<td>I,IV</td>
</tr>
<tr>
<td>anti-5-hydroxytryptamine (5-HT)</td>
<td>rabbit monoclonal (1:5000, ImmunoStar)</td>
<td>I, IV</td>
</tr>
<tr>
<td></td>
<td>rabbit polyclonal (1:2000, MP Biomedicals)</td>
<td>I</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-mouse-IgG</td>
<td>Alexa 488/568 conjugated (1:300-1:400, Molecular probes)</td>
<td>I, III, IV</td>
</tr>
<tr>
<td></td>
<td>HRP-conjugated (1:500, Amersham)</td>
<td>III</td>
</tr>
<tr>
<td>anti-rabbit-IgG</td>
<td>Alexa 488/568 conjugated (1:300-1:400, Molecular probes)</td>
<td>III,IV</td>
</tr>
<tr>
<td></td>
<td>HRP-conjugated (1:500, Chemicon)</td>
<td>IV</td>
</tr>
<tr>
<td>anti-sheep-IgG</td>
<td>Alexa 488/568 conjugated (1:300-1:400, Molecular probes)</td>
<td>IV</td>
</tr>
<tr>
<td><strong>TUNEL analyses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescein in situ detection kit</td>
<td>used according to manufacturer’s instructions (Roche)</td>
<td>III</td>
</tr>
<tr>
<td><strong>Counterstain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4′,6-diamino-2-phenylindole (DAPI)</td>
<td>included in the mounting media Vectashield (Vector)</td>
<td>I,III,IV</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

1. Microarray analysis of Fgfr1 CKO mutants (I-III)

To get better understanding of the Fgfr1 regulated and/or isthmic organizer specific genes, we used microarray-based strategies for identification of genes whose expression is affected in the conditional Fgfr1 mutants. For these studies, total RNA from dissected midbrain-R1 tissues of E10.5 wild-type and En1-Cre/+; Fgfr1Flox/Flox (Trokovic et al., 2003, referred as Fgfr1 CKO mutants hereafter) embryos was isolated. The dissected region contained the posterior midbrain as well as the most anterior part of the rhombomere 1 (R1) of the hindbrain (Fig. 12). The hybridizations were carried out on Affymetrix GeneChip arrays (mouse U74A version 2 arrays).

Among the genes, which were found to be down-regulated in the mutants, there were many whose expression was previously found to be down-regulated in Fgfr1 CKO mutants using the candidate gene approach (Table 5). Among the down-regulated transcripts, there were also products of many genes, which previously were not known to be expressed at the midbrain-R1 region or which represent previously poorly characterized transcripts (e.g. expressed sequence tags, ESTs; see Table 7). Many of the down-regulated genes are components or regulators of the signaling cascades downstream of receptor tyrosine kinases (Table 7 and Fig. 13).

Figure 12. Experimental design for microarray analysis of midbrain-R1 region. Reproduced from Jukkola et al. (2006).
Table 5. Genes downregulated in Fgfr1 CKO mutants at E10.5.

<table>
<thead>
<tr>
<th>Gene / EST</th>
<th>Affymetrix probe set ID</th>
<th>Fold change</th>
<th>Validation by in situ hybridisation*</th>
<th>Probe(s) for in situ hybridisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf17</td>
<td>98730_at</td>
<td>0.06</td>
<td>Fig. 1B in I</td>
<td>(Xu et al., 2000)</td>
</tr>
<tr>
<td>Spry1</td>
<td>163956_at</td>
<td>0.24</td>
<td>Fig. 1C in I</td>
<td>(Zhang et al., 2001)</td>
</tr>
<tr>
<td>En1</td>
<td>96253_at</td>
<td>0.29</td>
<td>Fig. 2A in I</td>
<td>(Davis and Joyner, 1988)</td>
</tr>
<tr>
<td>Fgf8</td>
<td>97742_s_at</td>
<td>0.30</td>
<td>Fig. 1B in I</td>
<td>(Crossley and Martin, 1995)</td>
</tr>
<tr>
<td>Spry2</td>
<td>116938_at</td>
<td>0.30</td>
<td>Fig. 1C in I</td>
<td>(Zhang et al., 2001)</td>
</tr>
<tr>
<td>Treh</td>
<td>102665_at</td>
<td>0.31</td>
<td>Fig. 2E in I</td>
<td>IMAGE 559851</td>
</tr>
<tr>
<td>Cnpy1</td>
<td>138472_at</td>
<td>0.35</td>
<td>Fig. 2C in I</td>
<td>IMAGE 559851</td>
</tr>
<tr>
<td>Fgf18</td>
<td>95316_at</td>
<td>0.35</td>
<td>Fig. 1B in I</td>
<td>IMAGE 651225</td>
</tr>
<tr>
<td>Fgfr1</td>
<td>97509_f_at</td>
<td>0.38</td>
<td>Expression downregulated</td>
<td>IMAGE 651225</td>
</tr>
<tr>
<td>Tnfrsf19 (Trade)</td>
<td>160670_at</td>
<td>0.39</td>
<td>Fig. 2K in I</td>
<td>(Xu et al., 2000)</td>
</tr>
<tr>
<td>Sef</td>
<td>133830_at</td>
<td>0.41</td>
<td>Fig. 1C in I</td>
<td>IMAGE 1178421</td>
</tr>
<tr>
<td>Erm (Etv5)</td>
<td>163173_at</td>
<td>0.41</td>
<td>Fig. 1D in I</td>
<td>IMAGE 3674281</td>
</tr>
<tr>
<td>Mkp3 (Dusp6)</td>
<td>93285_at</td>
<td>0.42</td>
<td>Fig. 1C in I</td>
<td>IMAGE 874051</td>
</tr>
<tr>
<td>Nfia</td>
<td>130461_at</td>
<td>0.48</td>
<td>In WT embryos: Broad expression in the midbrain-R1 region and in the neural tube Fig. 2F in I</td>
<td>IMAGE 1153158</td>
</tr>
<tr>
<td>Mrp4 (Abcc4)</td>
<td>111137_at</td>
<td>0.50</td>
<td></td>
<td>IMAGE 367701</td>
</tr>
<tr>
<td>Pax5</td>
<td>95890_r_at</td>
<td>0.50</td>
<td>Fig. 2D in I</td>
<td>IMAGE 333164</td>
</tr>
<tr>
<td>EST6</td>
<td>163120_at</td>
<td>0.51</td>
<td>In WT embryos: No specific expression detected</td>
<td>IMAGE 534449</td>
</tr>
<tr>
<td>Pcx</td>
<td>171076_i_at</td>
<td>0.51</td>
<td>In WT embryos: No specific expression detected</td>
<td>IMAGE 536361</td>
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<td>En2</td>
<td>98338_at</td>
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<td>Fig. 2B in I</td>
<td>IMAGE 5044506</td>
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<td>Sfp2</td>
<td>93503_at</td>
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<td>IMAGE 5053475</td>
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<td>Fgf15</td>
<td>97721_at</td>
<td>0.54</td>
<td>Fig. 1B in I</td>
<td>(McWhirter et al., 1997)</td>
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<tr>
<td>Atp1a1</td>
<td>93797_g_at</td>
<td>0.54</td>
<td>In WT embryos: No specific expression detected</td>
<td>UI-M-BH2.3-aqc-d-05-0-UI (BMAP)</td>
</tr>
<tr>
<td>4921506J03Rik</td>
<td>99163_at</td>
<td>0.54</td>
<td>In WT embryos: No specific expression detected</td>
<td>IMAGE 5721414</td>
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<tr>
<td>Igfbp5</td>
<td>100566_at</td>
<td>0.54</td>
<td>Fig. 2J in I</td>
<td>IMAGE 5290667</td>
</tr>
<tr>
<td>Nfib</td>
<td>160859_s_at</td>
<td>0.54</td>
<td>In WT embryos: Expressed in the ventral midbrain-R1 region</td>
<td>IMAGE 381625</td>
</tr>
<tr>
<td>RhoA (Arha2)</td>
<td>101112_g_at</td>
<td>0.55</td>
<td>In WT embryos: Broad expression in the midbrain-R1 region and in the CNS Fig. 21 in I</td>
<td>IMAGE 4190318</td>
</tr>
<tr>
<td>Sox3</td>
<td>92264_at</td>
<td>0.56</td>
<td>Fig. 21 in I</td>
<td>IMAGE 4234611</td>
</tr>
<tr>
<td>Drapc1</td>
<td>96132_at</td>
<td>0.59</td>
<td>Fig. 2M in I</td>
<td>IMAGE 368804</td>
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<tr>
<td>Flrt3</td>
<td>110370_at</td>
<td>0.60</td>
<td>Fig. 1C in I</td>
<td>IMAGE 1764103</td>
</tr>
<tr>
<td>Epes3 (Ftsj3)</td>
<td>95756_at</td>
<td>0.60</td>
<td></td>
<td>IMAGE 374528</td>
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<tr>
<td>Ccnd2</td>
<td>97504_at</td>
<td>0.60</td>
<td>Fig. 2H in I</td>
<td>IMAGE 5038498</td>
</tr>
<tr>
<td>Fahn</td>
<td>98967_at</td>
<td>0.62</td>
<td>In WT embryos: Expressed in the</td>
<td>IMAGE 5702881</td>
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</tbody>
</table>

**Results and Discussion**
Table 5 continuing

<table>
<thead>
<tr>
<th>Gene / EST</th>
<th>Affymetrix probe set ID</th>
<th>Fold change</th>
<th>Validation by in situ hybridisation*</th>
<th>Probe(s) for in situ hybridisation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eef1a1</td>
<td>94766_at</td>
<td>0.63</td>
<td>ventral midbrain</td>
<td></td>
</tr>
<tr>
<td>Pip92 (ler2)</td>
<td>99109_at</td>
<td>0.63</td>
<td>Expressed strongly in the ventral neural tube and in the MHB boundary region which is lost in mutants</td>
<td>IMAGE 3376243</td>
</tr>
<tr>
<td>Pea3 (Etv4)</td>
<td>92979_at</td>
<td>0.64</td>
<td>Fig. 1D in I (Lin et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>Kik1 (Hsd17b12)</td>
<td>94276_at</td>
<td>0.65</td>
<td>In WT embryos: no expression detected</td>
<td>IMAGE 6394399 IMAGE 747788 IMAGE 3492262 IMAGE 6812794</td>
</tr>
<tr>
<td>Snx5</td>
<td>117186_at</td>
<td>0.65</td>
<td>In WT embryos: no expression detected</td>
<td>IMAGE 671545</td>
</tr>
<tr>
<td>Tcf7</td>
<td>97994_at</td>
<td>0.65</td>
<td>Fig. 2N in I A gift from Irma Thesleff</td>
<td>IMAGE 1513890</td>
</tr>
<tr>
<td>Bnip3l</td>
<td>96255_at</td>
<td>0.66</td>
<td>In WT embryos: no expression detected</td>
<td>IMAGE 3492262 IMAGE 6812794</td>
</tr>
<tr>
<td>Gstm5</td>
<td>100629_at</td>
<td>0.67</td>
<td>In WT embryos: no expression detected</td>
<td>IMAGE 671545</td>
</tr>
<tr>
<td>Prkrir</td>
<td>99975_at</td>
<td>0.67</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ccnb1</td>
<td>160159_at</td>
<td>0.69</td>
<td>Expressed broadly in the CNS. Absent from the MHB border region and downregulated in the mutants</td>
<td>IMAGE 3971364 IMAGE 3973833 IMAGE 4014448</td>
</tr>
<tr>
<td>Spred2</td>
<td>161070_at</td>
<td>0.69</td>
<td>In WT embryos: no specific expression detected</td>
<td>IMAGE 4988566</td>
</tr>
<tr>
<td>Nrp</td>
<td>95016_at</td>
<td>0.69</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rraga</td>
<td>94257_at</td>
<td>0.70</td>
<td>In WT embryos: expressed broadly in the CNS and limb buds</td>
<td>IMAGE 5401569</td>
</tr>
<tr>
<td>1200007D18Rik</td>
<td>160184_at</td>
<td>0.71</td>
<td>In WT embryos: no specific expression detected</td>
<td>IMAGE 3494155</td>
</tr>
<tr>
<td>Pea15</td>
<td>100548_at</td>
<td>0.71</td>
<td>Expressed weakly in the ventral midbrain-R1 region which is lost in mutants</td>
<td>IMAGE 406749</td>
</tr>
<tr>
<td>CN04 (6430527G18Rik)</td>
<td>162901_i_at</td>
<td>0.71</td>
<td>In WT embryos: no specific expression detected at MHB</td>
<td>IMAGE 1245431</td>
</tr>
<tr>
<td>Sox21</td>
<td>167903_at</td>
<td>0.72</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>D7Wsu128e</td>
<td>103861_s_at</td>
<td>0.74</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Jmj</td>
<td>94341_at</td>
<td>0.74</td>
<td>Fig. 2G in I</td>
<td>IMAGE 6406875</td>
</tr>
</tbody>
</table>

* Published in I or if not stated refers to unpublished data.

The microarray screen revealed also genes, whose expression was up-regulated in the Fgfr1 CKO mutants (Table 6). Among these were many factors that have been associated with neuronal differentiation (see Table 8). In addition, the loss of isthmic signals can be expected to result in premature differentiation. Therefore, it was hypothesized that the up-regulated gene list may include novel components of the neuronal differentiation pathway at the midbrain-R1 region.
Table 6. Genes upregulated in Fgfr1 CKO mutants at E10.5.

<table>
<thead>
<tr>
<th>Gene / EST</th>
<th>Affymetrix probe set ID</th>
<th>Fold change</th>
<th>Validation by in situ hybridisation*</th>
<th>Probe for in situ hybridisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fst</td>
<td>98817_at</td>
<td>4.00</td>
<td>Fig. 3M, N in I</td>
<td>(Wang et al., 2004)</td>
</tr>
<tr>
<td>EST</td>
<td>166028_s_at</td>
<td>2.22</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tal-2</td>
<td>129118_at</td>
<td>2.00</td>
<td>In WT embryos: expressed in the tectum (Mori et al. 1999)</td>
<td></td>
</tr>
<tr>
<td>Mab21l1</td>
<td>165815_r_at</td>
<td>1.89</td>
<td>Fig. 3F in I</td>
<td>IMAGE 4526962</td>
</tr>
<tr>
<td>Edarb</td>
<td>163124_s_at</td>
<td>1.87</td>
<td>Fig. 3I in I</td>
<td>IMAGE 4971909</td>
</tr>
<tr>
<td>Math1</td>
<td>168404_at</td>
<td>1.84</td>
<td>Fig. 3D in I</td>
<td>IMAGE 4218223</td>
</tr>
<tr>
<td>EST</td>
<td>113895_at</td>
<td>1.77</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rgma</td>
<td>108717_at</td>
<td>1.66</td>
<td>Fig. 3C in I</td>
<td>IMAGE 1001290</td>
</tr>
<tr>
<td>EST</td>
<td>139205_at</td>
<td>1.66</td>
<td>Fig. 3K in I</td>
<td>IMAGE 2655939</td>
</tr>
<tr>
<td>Vtn</td>
<td>98549_at</td>
<td>1.65</td>
<td>Fig. 3A in I</td>
<td>IMAGE 5366291</td>
</tr>
<tr>
<td>Uncx4.1</td>
<td>92499_at</td>
<td>1.60</td>
<td>Fig. 3H in I</td>
<td>IMAGE 5716567</td>
</tr>
<tr>
<td>Ngfr (p75)</td>
<td>108762_at</td>
<td>1.57</td>
<td>Fig. 3G in I</td>
<td>(Qun et al., 1999)</td>
</tr>
<tr>
<td>EST</td>
<td>137358_at</td>
<td>1.55</td>
<td>Fig. 3L in I</td>
<td>IMAGE 3329477</td>
</tr>
<tr>
<td>Maf</td>
<td>115390_at</td>
<td>1.52</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EST</td>
<td>167322_at</td>
<td>1.52</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ckb</td>
<td>137242_f_at</td>
<td>1.49</td>
<td>Supp. Fig. 7J in I</td>
<td>IMAGE 6395794</td>
</tr>
<tr>
<td>Ptlnp</td>
<td>100927_at</td>
<td>1.46</td>
<td>Broad expression in the neural tube</td>
<td>IMAGE 4979759</td>
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<tr>
<td>Nlhh2</td>
<td>166814_f_at</td>
<td>1.44</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Wdfc1</td>
<td>166671_at</td>
<td>1.44</td>
<td>Fig. 3J in I</td>
<td>IMAGE 3823589</td>
</tr>
<tr>
<td>Tcpn1</td>
<td>108515_at</td>
<td>1.41</td>
<td>-</td>
<td>IMAGE 653006</td>
</tr>
<tr>
<td>Dach1</td>
<td>114999_at</td>
<td>1.41</td>
<td>Fig. 3E in I</td>
<td>IMAGE 6826750</td>
</tr>
</tbody>
</table>

* Published in I

Microarray data were categorized using gene ontology (GO) annotation. GO provides a controlled vocabulary organized in a hierarchical way that can be applied to describe gene products from mouse regarding: molecular function, biological process, and cellular component. The GO assignment relies on information from the AMIGO server http://www.geneontology.org/index.shtml. The GO classifications of downregulated and upregulated genes in the Fgfr1 CKO mutants at E10.5 are shown in tables 7 and 8, respectively. A summary of only the most specific GO annotations withdrawn from the AMIGO are listed in the tables 7 and 8. Fig. 13 shows that the representation of GO classes between up- and downregulated genes was quite different. The set of up-regulated genes was enriched with genes involved in intercellular signalling (e.g. secreted proteins) and neuronal transcription factors but lacks genes involved in signal transduction (intracellular), whereas genes involved in responses to external stimulus (growth factors; MAPK pathway), cell adhesion, and cell proliferation were overrepresented among down-regulated genes.
Table 7. Functional classification of downregulated genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>GO Biological Process Description</th>
<th>GO Molecular Function Description</th>
<th>GO Cellular Component Description</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf17</td>
<td>fibroblast growth factor 17</td>
<td>signal transduction cell-cell signaling nervous system development</td>
<td>fibroblast growth factor receptor binding growth factor activity</td>
<td>extracellular space</td>
<td>0.06</td>
</tr>
<tr>
<td>Spry1</td>
<td>sprouty homolog 1 (Drosophila)</td>
<td>ureteric bud development induction of an organ regulation of signal transduction negative regulation of MAPK activity</td>
<td>protein binding</td>
<td>cytoplasm membrane</td>
<td>0.24</td>
</tr>
<tr>
<td>En1</td>
<td>engrailed 1</td>
<td>regulation of transcription, DNA-dependent potassium ion transport dorsal/ventral pattern formation neuron differentiation midbrain-hindbrain boundary development</td>
<td>DNA binding transcription factor activity voltage-gated potassium channel activity sequence-specific DNA binding</td>
<td>nucleus voltage-gated potassium-channel complex</td>
<td>0.29</td>
</tr>
<tr>
<td>Fgf8</td>
<td>fibroblast growth factor 8</td>
<td>regulation of progression through cell cycle MAPKKK cascade signal transduction positive regulation of cell proliferation negative regulation of apoptosis generation of neurons</td>
<td>fibroblast growth factor receptor binding growth factor activity</td>
<td>extracellular space</td>
<td>0.3</td>
</tr>
<tr>
<td>Spry2</td>
<td>sprouty homolog 2 (Drosophila)</td>
<td>cell-cell signaling development regulation of signal transduction MAPK activity cell fate commitment</td>
<td>protein binding</td>
<td>microtubule membrane</td>
<td>0.3</td>
</tr>
<tr>
<td>Trh</td>
<td>thyrotropin releasing hormone</td>
<td>signal transduction cell-cell signaling hormone-mediated signaling</td>
<td>hormone activity neuroepitope hormone activity protein binding thyrotropin-releasing hormone activity</td>
<td>extracellular space soluble fraction</td>
<td>0.31</td>
</tr>
<tr>
<td>Cnpy1</td>
<td>Canopy 1 homolog (Zebrafish) (expressed sequence AI853839)</td>
<td>fibroblast growth factor receptor signalling pathway midbrain-hindbrain organizer development</td>
<td>---</td>
<td>endoplasmic reticulum</td>
<td>0.35</td>
</tr>
<tr>
<td>Fgf18</td>
<td>fibroblast growth factor 18</td>
<td>signal transduction cell-cell signaling positive regulation of cell proliferation</td>
<td>fibroblast growth factor receptor binding growth factor activity</td>
<td>extracellular space nucleolus</td>
<td>0.35</td>
</tr>
<tr>
<td>Fgfr1</td>
<td>fibroblast growth factor receptor 1</td>
<td>MAPKKK cascade protein amino acid phosphorylation signal transduction brain development regulation of cell proliferation cell growth negative regulation of apoptosis</td>
<td>protein kinase activity protein serine/threonine kinase activity protein tyrosine kinase activity receptor activity heparin binding</td>
<td>extracellular space membrane fraction integral to plasma membrane</td>
<td>0.38</td>
</tr>
<tr>
<td>Tnfrsf19</td>
<td>tumor necrosis factor receptor superfamily, member 19</td>
<td>induction of apoptosis JNK cascade</td>
<td>receptor activity tumor necrosis factor receptor activity protein binding</td>
<td>extracellular space integral to membrane</td>
<td>0.39</td>
</tr>
<tr>
<td>Sef</td>
<td>Similar expression to Fgfs</td>
<td>dorsal/ventral pattern specification negative regulation of FGF receptor signalling pathway</td>
<td>transmembrane receptor activity</td>
<td>integral to membrane</td>
<td>0.41</td>
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<tr>
<td>Erm (or Etv5)</td>
<td>ets variant gene 5 etv variant gene 5 pseudogene</td>
<td>regulation of transcription, DNA-dependent organ morphogenesis positive regulation of transcription</td>
<td>DNA binding transcription factor activity transcriptional activator activity</td>
<td>nucleus</td>
<td>0.41</td>
</tr>
<tr>
<td>Mkp3 (or Dusp6)</td>
<td>dual specificity phosphatase 6</td>
<td>protein amino acid dephosphorylation protein amino acid dephosphorylation</td>
<td>phosphoprotein MAP kinase phosphatase activity, tyrosine/serine/threonine phosphatase activity protein binding hydrophosphatase activity</td>
<td>---</td>
<td>0.42</td>
</tr>
<tr>
<td>Nfia</td>
<td>nuclear factor IIA</td>
<td>DNA replication regulation of transcription, DNA-dependent electron transport</td>
<td>DNA binding transcription factor activity iron ion binding electron carrier activity</td>
<td>intracellular nucleus</td>
<td>0.48</td>
</tr>
<tr>
<td>Mrp4 (or Abcc4)</td>
<td>ATP-binding cassette, sub-family C (CFTR/ABCC), member 4</td>
<td>ion transport multidrug transport</td>
<td>nucleotide binding chloride channel activity ATP binding multidrug efflux pump activity ATPase activity</td>
<td>integral to membrane</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Table 7 continuing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Regulation/Description</th>
<th>Protein/DNA Binding</th>
<th>Cell Location</th>
<th>Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax5</td>
<td>regulation of transcription, DNA-dependent transcription from RNA polymerase II promoter nervous system development</td>
<td>DNA binding transcription factor activity protein binding</td>
<td>nucleus transcription factor complex</td>
<td>0.5</td>
</tr>
<tr>
<td>E5T6</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.51</td>
</tr>
<tr>
<td>Pcx</td>
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<td>---</td>
<td>---</td>
<td>0.51</td>
</tr>
<tr>
<td>En2</td>
<td>regulation of transcription, DNA-dependent development neuron differentiation midbrain- hindbrain boundary development</td>
<td>DNA binding transcription factor activity sequence-</td>
<td>nucleus</td>
<td>0.52</td>
</tr>
<tr>
<td>Sfrp2</td>
<td>secreted frizzled-related protein 2</td>
<td>transmembrane receptor activity</td>
<td>membrane</td>
<td>0.52</td>
</tr>
<tr>
<td>Fgf15</td>
<td>fibroblast growth factor 15</td>
<td>fibroblast growth factor receptor binding growth factor activity</td>
<td>extracellular space</td>
<td>0.54</td>
</tr>
<tr>
<td>Atp1a1</td>
<td>ATPase, Na+/K+ transporting, alpha 1 polypeptide</td>
<td>ion transport cation transport potassium ion transport sodium ion transport monovalent inorganic cation transport ATP hydrolysis coupled proton transport metabolism</td>
<td>nucleotide binding magnesium ion binding catalytic activity sodium:potassium-exchanging ATPase activity transmembranous movement of ions, phosphorylative mechanism</td>
<td>membrane fraction integral to plasma membrane sodium:potassium-exchanging ATPase complex</td>
</tr>
<tr>
<td>4921506J03R</td>
<td>RIKEN cDNA 4921506J03 gene</td>
<td>---</td>
<td>---</td>
<td>0.54</td>
</tr>
<tr>
<td>Igfbp5</td>
<td>insulin-like growth factor binding protein 5</td>
<td>regulation of cell growth signal transduction</td>
<td>insulin-like growth factor binding</td>
<td>extracellular space</td>
</tr>
<tr>
<td>Nfib</td>
<td>nuclear factor I/B</td>
<td>DNA replication regulation of transcription, DNA-dependent negative regulation of cell proliferation hindbrain development</td>
<td>DNA binding transcription factor activity</td>
<td>intracellular nucleus</td>
</tr>
<tr>
<td>Rhoa</td>
<td>ras homolog gene family, member A, similar to aplysia ras-related homolog A2 similar to aplysia ras-related homolog A2</td>
<td>cell morphogenesis cell adhesion cell-matrix adhesion small GTPase mediated signal transduction actin cytoskeleton organization and biogenesis cell differentiation positive regulation of NF-kappaB import into nucleus negative regulation of neuron apoptosis</td>
<td>nucleotide binding GTPase activity signal transducer activity</td>
<td>intracellular nucleus cytoskeleton plasma membrane</td>
</tr>
<tr>
<td>Sox3</td>
<td>SRY-box containing gene 3</td>
<td>establishment and/or maintenance of chromatin architecture regulation of transcription, DNA-dependent central nervous system development forebrain development</td>
<td>DNA binding transcription factor activity</td>
<td>nucleus transcription factor complex</td>
</tr>
<tr>
<td>Drapcl (or Apcdd1)</td>
<td>adenomatosis polyposis coli down-regulated 1</td>
<td>---</td>
<td>---</td>
<td>integral to membrane</td>
</tr>
<tr>
<td>Fln3</td>
<td>fibronectin leucine rich transmembrane protein 3</td>
<td>---</td>
<td>protein binding</td>
<td>integral to membrane</td>
</tr>
<tr>
<td>Epc3 (or Pts3)</td>
<td>FtsI homolog 3 (E. coli)</td>
<td>rRNA processing</td>
<td>methyltransferase activity transerase activity</td>
<td>nucleus</td>
</tr>
<tr>
<td>Ccnd2</td>
<td>cyclin D2</td>
<td>regulation of progression through cell cycle</td>
<td>protein binding cyclin-dependent protein kinase regulator activity</td>
<td>nucleus</td>
</tr>
<tr>
<td>Fabp7</td>
<td>fatty acid binding protein 7, brain</td>
<td>transport</td>
<td>binding lipid binding</td>
<td>cell projection cell soma</td>
</tr>
<tr>
<td>Eef1a1</td>
<td>eukaryotic translation elongation factor 1 alpha 1</td>
<td>protein biosynthesis transaltional elongation</td>
<td>translation elongation factor activity nucleotide binding GTP binding</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>Pip92 (or ler2)</td>
<td>immediate early response 2</td>
<td>---</td>
<td>---</td>
<td>cytoplasm</td>
</tr>
</tbody>
</table>
Table 7 continuing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Function</th>
<th>Localization</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pea3</strong></td>
<td>ets variant gene 4 (E1A enhancer binding protein, E1AF)</td>
<td>regulation of transcription, DNA-dependent motor axon guidance positive regulation of transcription</td>
<td>DNA binding transcription factor activity</td>
<td>nucleus</td>
</tr>
<tr>
<td><strong>Kiki1</strong></td>
<td>hydroxysteroid (17-beta) dehydrogenase 12</td>
<td>steroid biosynthesis metabolism lipid biosynthesis</td>
<td>estradiol 17-beta dehydrogenase activity</td>
<td>endoplasmic reticulum integral to membrane</td>
</tr>
<tr>
<td><strong>Snx5</strong></td>
<td>sorting nexin 5</td>
<td>transport cell communication protein transport</td>
<td>protein binding phosophoinositide binding</td>
<td>---</td>
</tr>
<tr>
<td><strong>Tcf7</strong></td>
<td>transcription factor 7, T-cell specific</td>
<td>regulation of transcription, DNA-dependent Wnt receptor signaling pathway regulation of cell proliferation regulation of apoptosis</td>
<td>DNA binding transcription factor activity</td>
<td>nucleus transcription factor complex</td>
</tr>
<tr>
<td><strong>Bnip3l</strong></td>
<td>BCL2/adenovirus E1B interacting protein 3-like</td>
<td>induction of apoptosis negative regulation of survival gene product activity</td>
<td>protein binding</td>
<td>nucleus mitochondrial integral to membrane</td>
</tr>
<tr>
<td><strong>Gstm5</strong></td>
<td>glutathione S-transferase, mu 5</td>
<td>establishment of blood-nerve barrier metabolism</td>
<td>glutathione transferase activity</td>
<td>---</td>
</tr>
<tr>
<td><strong>Prkrir</strong></td>
<td>protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (PS8 repressor)</td>
<td>regulation of translation signal transduction negative regulation of cell proliferation</td>
<td>DNA binding protein binding zinc ion binding metal ion binding protein dimerization activity</td>
<td>nucleus</td>
</tr>
<tr>
<td><strong>Ccnb1</strong></td>
<td>cyclin B1, related sequence 1 cyclin B1</td>
<td>regulation of progression through cell cycle mitosis cytokinesis protein targeting</td>
<td>cyclin-dependent protein kinase regulator activity protein binding kinase activity</td>
<td>nucleus</td>
</tr>
<tr>
<td><strong>Spred2</strong></td>
<td>sprouty-related, EVH1 domain containing 2</td>
<td>inactivation of MAPK activity regulation of signal transduction</td>
<td>stem cell factor receptor protein binding</td>
<td>plasma membrane cytoplasmic vesicle</td>
</tr>
<tr>
<td><strong>Nrp1</strong></td>
<td>neuropilin 1</td>
<td>cell adhesion signal transduction cell-cell signaling nervous system development positive regulation of cell proliferation</td>
<td>receptor activity semaphorin receptor activity</td>
<td>membrane fraction endoplasmic reticulum integral to membrane</td>
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<tr>
<td><strong>Rraga</strong></td>
<td>Ras-related GTP binding A</td>
<td>apoptosis regulation of cytolysis</td>
<td>nucleotide binding protein binding GTP binding protein homodimerization activity protein heterodimerization activity</td>
<td>nucleus cytoplasm</td>
</tr>
<tr>
<td><strong>1200007D18 Rik</strong></td>
<td>endoplasmic reticulum-golgi intermediate compartment (ERGIC) 1</td>
<td>transport ER to Golgi vesicle-mediated transport transport</td>
<td>protein binding</td>
<td>endoplasmic reticulum ER-Golgi intermediate compartment integral to membrane</td>
</tr>
<tr>
<td><strong>Pea15</strong></td>
<td>phosphoprotein enriched in astrocytes 15</td>
<td>transport regulation of apoptosis</td>
<td>protein kinase C binding protein binding</td>
<td>membrane fraction cytosol microtubule associated complex</td>
</tr>
<tr>
<td><strong>CN04 6430527G18 Rik</strong></td>
<td>RIKEN cDNA 6430527G18 gene</td>
<td>---</td>
<td>protein binding zinc ion binding metal ion binding protein</td>
<td>nucleus</td>
</tr>
<tr>
<td><strong>Sox21</strong></td>
<td>SRY-box containing gene 21</td>
<td>transcription regulation of transcription, DNA-dependent</td>
<td>DNA binding transcription factor activity</td>
<td>chromatin nucleus</td>
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<tr>
<td><strong>D7Wsu128e</strong></td>
<td>DNA segment, Chr 7, Wayne State University 128, expressed</td>
<td>protein modification</td>
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<tr>
<td><strong>Jarid2 (or Jmj)</strong></td>
<td>jumonji, AT rich interactive domain 2</td>
<td>negative regulation of transcription from RNA polymerase II promoter regulation of transcription, negative regulation of cell proliferation</td>
<td>DNA binding protein binding transcriptional repressor activity</td>
<td>intracellular nucleus</td>
</tr>
</tbody>
</table>
Table 8. Functional classification of upregulated genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>GO Biological Process Description</th>
<th>GO Molecular Function Description</th>
<th>GO Cellular Component Description</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fst</td>
<td>follistatin</td>
<td>BMP signaling pathway pattern specification</td>
<td>protein binding activin inhibitor activity</td>
<td>extracellular region extracellular space</td>
<td>4</td>
</tr>
<tr>
<td>2610034M16Rik</td>
<td>RIKEN cDNA 2610034M16 gene</td>
<td>protein targeting</td>
<td>protein binding</td>
<td>---</td>
<td>2.22</td>
</tr>
<tr>
<td>Tal-2</td>
<td>T-cell acute lymphocytic leukemia</td>
<td>regulation of transcription, DNA-dependent</td>
<td>DNA binding transcription regulator activity</td>
<td>nucleus</td>
<td>2</td>
</tr>
<tr>
<td>Mab21f1</td>
<td>mab-21-like 1 (C. elegans)</td>
<td>positive regulation of cell proliferation protein localization</td>
<td>protein kinase binding protein kinase A binding</td>
<td>Golgi trans face cytosol plasma membrane</td>
<td>1.89</td>
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**Figure 13.** A summary of molecular functions (GO) of differentially expressed genes. (A) downregulated genes; (B) upregulated genes.
To validate our microarray results, this analysis was followed by a whole-mount mRNA *in situ* hybridization analysis. First, we concentrated on expression analysis of a down-regulated gene list showed in Table 5. Initially this list produced by the microarray analysis consists of sets of DNA probes, each chosen to record expression of specific genes. In Affymetrix GeneChip methodology, the set of probes relating to a gene is referred to as a probeset, and the sequence which is best associated with the transcribed region being interrogated by the probeset is referred to as its representative sequence (Liu et al., 2003a). The sequence annotations referring to the information about the representative sequence for a probeset are presented in the Affymetrix NetAffx service (http://www.affymetrix.com). This tool was utilized to locate the probes within the mouse genome and the corresponding cDNAs were ordered from the IMAGE consortium. These plasmids were then used to produce the *in situ* hybridization probes for consequent validation experiments.

## 2. Identification of FGF regulated genes at the midbrain-R1 region (I, III and unpublished)

In addition to the components of the FGF-FGFR signal transduction pathway (see section 2.1.1.) our microarray screen identified several other genes being differentially expressed in the *Fgfr1* mutant embryos (see sections 2.1.2. and 2.1.3.). Comparison of the midbrain-R1 tissues from E10.5 wild-type and *Fgfr1* mutant embryos identified 51 down-regulated and 20 up-regulated genes, respectively (see Tables 5 and 6). The downregulated list included genes involved in the patterning of midbrain and R1, MHB-specific genes, cell-cycle regulators, and components of various signal transduction cascades (Table 7 and Fig. 13).

### 2.1. Down-regulated genes (I, III and unpublished)

#### 2.1.1. Down-regulation of FGF-FGFR signaling components at E10.5 (I,III)

The down-regulated genes identified by microarray screen included several known members of the FGF signal transduction pathway (Table 5). These included FGF ligands, signal regulators and nuclear effectors. At E9.5 the expression of Fgf8/17/18 was not down-regulated in the *Fgfr1* mutant embryos. However, comparison of expression levels by microarray analysis showed that Fgf8/15/17/18 were down-regulated in E10.5 *Fgfr1* mutant mice (Table 5). This was confirmed by *in situ* hybridization analysis. Expression of Fgf17 was completely abolished from the midbrain-R1 region whereas Fgf8 and Fgf18 expression was maintained in the most ventral region. This suggests, that other FGF receptors (FGFR2 and 3) are mediating isthmic FGF signals in the *Fgfr1* mutants (see below).

Consistent with this, the most evident downregulation of Fgf15 was observed in the lateral midbrain, whereas the ventral Fgf15 expression domain was unchanged or even upregulated. Sef (similar expression to Fgfs) is a receptor molecule that is associated with the FGF/Ras-MAPK signaling. At E10.5 wild-type embryos, Sef expression was detected in the midbrain-R1 region and extended posterior to the isthmus. In the mutant E10.5 embryos, a down-regulation of Sef was seen in the most dorsal part of the midbrain-R1 region and extended posterior to the isthmus. In the mutant E10.5 embryos, a down-regulation of Sef was seen in the most dorsal part of the midbrain-R1 region. Similarly to Pea3 and Erm at E9.5, Sef expression was also downregulated close to the border between midbrain and R1. At E10.5, expression of
Erm and Pea3 was clearly down-regulated at the midbrain-R1 region in the Fgfr1 mutant embryos compared to the wild-type embryos. However, in the Fgfr1 mutants, some expression of Erm and Pea3 was still detected in the ventral midbrain-R1 region.

Sprouty (or SPRY) and MKP3 proteins act as negative regulators of the FGF/Ras-MAPK signaling pathway. Both Spry1 and Spry2 are expressed at the midbrain-R1 region at E10.5. In the Fgfr1 mutant embryos, Spry1 transcripts were abolished from the dorsal midbrain-R1 domain, but remained in the ventral midbrain-R1 region. By contrast, expression of Spry2 and Mkp3 was down-regulated throughout the midbrain-R1 region in the Fgfr1 mutant mice at E10.5 compared to wild-type embryos. Although, Mkp3 expression was clearly down-regulated at E10.5 in Fgfr1 mutants, some expression was detected in the most ventral midbrain-R1 domain when whole-mount embryos were sectioned after in situ hybridizations. A positive feedback loops provides a basis for the formation and maintenance of local organizing centers. In Xenopus and zebrafish, expression of Mkp3 has been shown to induce by RA and β-catenin pathways (Tsang and Dawid, 2004; Tsang et al., 2004). In Fgfr1 mutants, Mkp3 expression was lost from the dorsal midbrain-R1 region already at E9.5. Interestingly, at this stage, Wnt1 and Drapc1 expression is still detected in the dorsal region. Thus, it can be speculated that FGF8/17/18 are the key molecules which are regulating the MAPK-RAS pathway, which in turn is required for the dorsal patterning of R1.

Recent studies have shown that rat and Xenopus Flrt3 genes are coexpressed with Fgfs (Bottcher et al., 2004; Lacy et al., 1999; Robinson et al., 2004). We detected Flrt3 expression in wild-type embryos from E10.5 onwards at the narrow midbrain-R1 region. In the Fgfr1 mutant embryos, Flrt3 expression domain at the midbrain-R1 region was abolished. The lack of Flrt3 expression in the boundary cells between the midbrain and R1 may be the mechanism to the observed tissue segregation in Fgfr1 mutants. Thus,

Figure 14. Cnpy1 is specifically expressed in the midbrain-R1 region. Expression pattern of Cnpy1 is conserved between mouse (A) and zebrafish (B). Arrowheads point to the IsO and an arrow marks the more posterior expression of Cnpy1. hpf, hours post fertilization; ss, somite stage.
similarly than WNT signaling modulates cadherin-mediated cell adhesion in Drosophila imaginal disc cells (Wodarz et al., 2006), the FGFR signaling is needed for the homophilic FLRT-mediated cell-cell adhesion. In the Fgfr1 mutants the dorsal patterning was more affected. Thus, it is possible that residual FGFR signaling may be required for maintaining the FLRT-mediated adhesion in the ventral region. In addition to Flrt3, Flrt1 and Flrt2 are expressed in the ventral midbrain-R1 region as showed by (Haines et al., 2006). In Fgfr1 mutants, expression of Flrt1 or Flrt2 was not studied.

Thus, validating our microarray approach, known members of the FGF synexpression group and components of the FGF signal pathway were identified as being down-regulated in the Fgfr1 mutants at E10.5. Taken together, most of these FGF-FGFR1 signaling components described here retained expression in the ventral midbrain-R1 region in the Fgfr1 mutants indicating the presence of residual FGF signaling.

2.1.2. Cnpy1 as a novel regulator of FGF signaling at the midbrain-R1 region (I and unpublished)

Expression of Iso regulated transcription factors En1/2 and Pax5 was abolished at the dorsal part of the midbrain-R1 region of mutant embryos. Interestingly, previously uncharacterized gene (NM_...
The role of *Cnpy1* in the maturation of ventral DA neurons might be less obvious. However, further functional analyses of mouse *Cnpy1* are needed to clarify its role in the FGF-FGFR regulated patterning and differentiation in the midbrain-R1 region.

### 2.1.3. Down-regulated genes expressed as a narrow stripe near the MHB (I and unpublished)

Genes that were identified to be expressed in a narrow stripe near the MHB included *Trh, Mrp4, Igfbp5* and *Jumonji (Jmj)*. The role of *Jmj* in the midbrain-R1 region is discussed in section 5.4. *Trh* and *Mrp4* showed specific expression in the midbrain-R1 region whereas *Igfbp5* expression was detected in various brain areas.
regions between E9.5 and E11.5. The importance of these genes during the midbrain-R1 development has not been reported. Mouse knockouts of \textit{Trh}, \textit{Mrp4}, \textit{Igfbp5} and \textit{Jumonji} has been generated, but only \textit{Jmj} knockout mice show obvious brain phenotype (Assem et al., 2004; Lai and Tan, 2002; Motoyama et al., 1997; Ning et al., 2007; Takeuchi et al., 1995; Takeuchi et al., 1999; Urayama et al., 2002; Yamada et al., 1997). We studied the expression of early patterning genes in \textit{Trh} KO embryos. At E10.5, expression of the genes studied was unchanged in the \textit{Trh} KO embryos compared with the wild-type (+/+ ) litter mates (Fig. 17). At the later stage (E15.5), as well, no obvious changes in gene expression patterns of \textit{Otx2}, \textit{En1} or \textit{Drapc1} were detected (Fig. 18).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure17.png}
\caption{Expression of patterning genes in the \textit{Trh} KO at E10.5.} \textit{Radioactive in situ hybridization analysis with the probes indicated (A-F). Lines mark the most posterior \textit{Otx2} expression boundary. mb, midbrain; r1, rhombomere 1.}
\end{figure}
2.1.4. Other down-regulated genes in the Fgfr1 CKO mutants (I)

Among the other down-regulated genes (Table 5) we observed Cyclins (CyclinD1/D2/B1) and Sox3 and Sox21, which were broadly expressed in the dorsal midbrain and hindbrain. Interestingly, a narrow domain of Cyclins- and Sox3-negative cells was detected in the border between mid- and hindbrain. Expression of these genes was downregulated in the dorsal midbrain-R1 region of Fgfr1 mutant embryos. It has been suggested that expression of Sox genes is dependent of FGF signaling (Takemoto et al., 2006). In addition, Sox3 expression is inhibited in the neuroepithelial boundary regions to prevent the early exit from the cell cycle (Rizzoti et al., 2004). Thus, these cellular mechanisms linking FGF signaling to proliferation and differentiation prompted us to study the genes important for the neuronal differentiation in the vicinity of the MHB (see section 3 below).

2.2. Up-regulated genes show loss of expression gradients in Fgfr1 CKO mutant embryos (I)

Taken together, the majority of upregulated genes (Table 6) showed mainly two main types of expression in wild-type embryos - dorsal gradients or combined dorsal and ventral gradients. Genes such as Fst, Vtn, Wfde1, RgmA, Math1, Dach1 and Mab21l1 were expressed as dorsal gradients decreasing towards the MHB in wild-type embryos. In Fgfr1 mutants, the midbrain and R1 gradients were lost and the expression continued uniformly strong towards the boundary or across it. This effect was the most prominent for Fst, which is a known BMP signaling component.

Figure 18. Expression of patterning genes in the Trh KO at E15.5. Radioactive in situ hybridization analysis with the probes indicated (A-C). Lines mark the most posterior Otx2 expression boundary. cb, cerebellum; mb, midbrain.
In summary, several upregulated genes showed marked similarity in their expression patterns in E10.5 embryos. Most of them were expressed as gradients decreasing towards MHB, both in midbrain and rhombomere 1. In Fgfr1 mutant embryos, these gradients disappeared both in dorsal and in ventral regions.

BMPs are thought to regulate the development of the LC. Thus, to address the role of follistatin (FST) on neurogenesis, the expression of Phox2a and Ngn2 were analyzed on E10.5 Fst knock-out embryos (Matzuk et al., 1995). The expression of these both genes in mutants was, however, identical to the wild-type controls and no changes in the LC were seen at this stage. In addition, there were no observable changes in the appearance of locus coeruleus, SA neurons or midbrain DA neurons in Fst mutants at E14.5. This suggests that the upregulation of Fst alone would not lead to notable changes in neurogenesis. In addition, spreading of Fst expression anteriorly in Fgfr1 mutant embryos does not prevent the development of LC.

3. Neuronal progenitor cell populations are affected in Fgfr1 CKO mutant embryos (I)

3.1. Neuronal progenitor cell populations are affected in Fgfr1 CKO mutant embryos (I)

Since our results suggested loss of neuronal differentiation gradients in Fgfr1 mutants, we wanted to know what effects FGFR1 conditional inactivation would have on different subsets of neuronal progenitor cells in the midbrain-R1 region. We analyzed several genes which are known to be expressed in different neuronal progenitors at certain stages of development.

In the Fgfr1 mutant embryos, the Ngn2 expressing cell populations of the ventral midbrain and hindbrain were less compact and spread towards the boundary (E10.5 and E11.5). Especially, a strongly expressing cell population near the MHB in ventral R1 showed an extensive scattering effect. Also the LC domain in E10.5 mutants seemed less compact as compared to the wild-type. The III and IV cranial ganglia were the same size in the Fgfr1 mutants as in the wild-type embryos as marked by the Phox2a expression. However, Phox2a expression domains appeared to be fused together, presumably as a result of the loss of the boundary cell population between mid- and hindbrain.

As the development of III and IV cranial ganglia as well as LC is initiated earlier compared to other neuronal populations, such as SA or DA neurons, they can be less dependent on the isthmic signaling. Thus, the major events concerning their formation take place before the need for FGFR1 function. This would explain their mild phenotype in Fgfr1 conditional mutant adults - III and IV ganglia appear normal, and LC is somewhat disorganized (Trokovíc et al., 2003).

To study the fate of the neuronal precursor cells in Fgfr1 mutants, we analyzed the expression of Hes3, a bHLH transcriptional factor and a known Notch effector, which is inhibiting neuronal differentiation in the MHB (Hirata et al., 2001). In E10.5 Fgfr1 mutant embryos, the expression of Hes3 was downregulated but was still weakly detectable in the caudal midbrain near the IsO. The residual expression in the R1 near the IsO was confined to the neuroectoderm. Thus the loss of Hes3 expression supports the
results obtained from the upregulated genes (see above), which suggested that neuronal differentiation was shifted towards the MHB region in Fgfr1 mutant embryos.

In wild type embryos, a gap of mature neurons was evident near the MHB region, as seen with anti-TUJ1 staining. In Fgfr1 mutants the gap disappeared. The number of radial glia cells and mitotic cells outside the boundary region, as determined by anti-RC2 and anti-PH3 immunostainings, respectively, was not significantly altered in Fgfr1 mutants (data not shown). Earlier quantification studies did not show any changes in cell proliferation and apoptosis in the ventral MHB region (Trokovic et al., 2003). However, the changes in the ratio of progenitor neurons to mature neurons might be too subtle to be easily detectable in these early embryonic stages.

3.2. Midbrain dopaminergic neuron progenitor pool spreads in Fgfr1 CKO mutants (I)

Since FGF is thought to control the development of DA neurons in the ventral midbrain, we examined what happens to the DA neuron progenitors in the midbrain. For this, we analyzed the expression of several genes known to be involved in the early development of midbrain DA neurons and quantified the neurons in E15.5 Fgfr1 mutant and wild-type embryos.

In E11.5 Fgfr1 mutant embryos, the expression of Nurr1, Pitx3 and Aldh1 (also known as Aldh1a1 and Raldh1) was expanded posteriorly towards the border between midbrain and R1. Then we analyzed the tyrosine hydroxylase (TH) positive dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra at E15.5 wild-type and Fgfr1 mutant embryos. The comparison between the total numbers of DA neurons showed not statistically significant difference between the wild-type and mutant embryos. Next, we looked at the differences between the caudal and rostral DA populations as these were compared individually. The analysis revealed an increase in the caudal population and conversely a decrease in the rostral population of DA neurons in the Fgfr1 mutant embryos. Thus, consistent with observed changes in gene expression patterns of DA markers TH immunostaining at E15.5 stage revealed a caudal shift in the position of DA neurons in Fgfr1 mutants. Thus, it seems that although the area where the DA neurons are located expands in mutants, the total number of these neurons does not change. The caudal shift is probably due to the caudal spreading of Otx2 expression. In ventral midbrain, Otx2 expression is scattered in Fgfr1 mutants at E15.5, and the boundary between Otx2 positive and negative areas is less obvious than in the wild-type. This would suggest that the mixing of ventral midbrain and R1 cells forms a tissue mosaic in the Fgfr1 mutants. A similar phenomenon has been observed in zebrafish Ace mutants where R1 is transformed into midbrain in the absence of Fgf8 from the IsO (Jaszai et al., 2003). However, in Fgfr1 mutants, instead of complete fate transformation of R1 an extensive mixing of ventral midbrain cells into R1 is observed.

3.3. The most rostral serotonergic neuron precursors are lost in Fgfr1 CKO mutant embryos (I)

FGF8 from the IsO is thought to be one of the major signals guiding the development of serotonergic (SA) neurons in the raphe nuclei (Ye et al., 1998). To find out whether
precursor of SA neurons are affected in *Fgfr1* mutant embryos, we analyzed the expression patterns of several genes that are known to be involved in the SA neuron specification and quantified the rostral SA neurons in *Fgfr1* mutant and wild-type embryos at E15.5.

In *Fgfr1* mutants, several genes which are involved in the specification of SA neurons, such as *Gata3, Mash1/Ascl1* and *Pet1* were downregulated near the midbrain-R1 region in the ventral R1. Next we performed anti-5HT immunohistochemistry on E11.5 and E15.5 sagittal sections to characterize whether downregulation of the transcription factors has an effect on SA neuron development. At E11.5 in wild-type embryos, 5-HT was detected in the same area as *Pet1*. In *Fgfr1* mutants, 5-HT expression was abolished near the boundary region, similarly to *Pet1*. At 15.5, when all SA neurons have developed in mouse, 5-HT could be seen in a large area extending to the top of the mesencephalic flexure in the wild-type. In *Fgfr1* mutants, the rostral boundary of SA neuron population was shifted caudally and almost no 5-HT positive neurons were seen near the top of the mesencephalic flexure. The SA neurons located more caudally seemed unaffected in mutants.

When the total number of neurons was counted and compared, their number was significantly decreased in mutants. The neurons were divided into dorsal and ventral populations which were compared individually (similarly to the DA neurons). The loss of the most rostral SA neurons explains the decrease in the dorsal population. The ventral population seems to be unaffected in *Fgfr1* mutants. The quantification results reflect the changes in early gene expression, where the downregulation was seen in the rostral area only.

Taken together, it is interesting that the downregulation of transcription factors *Mash1, Gata3* and *Pet1* could be seen only in the most anterior R1. The early downregulation of the proneural gene *Mash1* suggests that the SA progenitors near the MHB require a high dose of FGF-signals from the IsO in order to develop normally. In the explant culture experiments, FGF4 in combination with FGF8 and SHH could induce SA neuron development (Ye et al., 1998). However, FGF4 itself is not expressed in the vicinity of developing rostral SA neurons at E10.5-11.5. It has been suggested that FGF4 expressed in the primitive streak primes neuroectoderm for the development of SA precursors (Ye et al., 1998). However, since FGFR1 is inactivated by 10 somite stage in our conditional mutants (Trokovic et al., 2003), the early FGF4 signaling functions normally in *Fgfr1* CKO mutants. This suggests that other FGFs from the IsO are the major molecular regulators in SA neuron development. Thus, it is possible that in the explant culture experiments FGF4 mimicked the high FGF signaling activity near the MHB, which is normally achieved by the combination FGF8/FGF17/FGF18 molecules expressed by the IsO. In *Fgfr1* mutants, the amount of activating isthmic signals is reduced at E10.5 and the SA neuron developmental pathway is impaired. Serotonergic neurons further away from the boundary express normally all the genes in the SA pathway, as well as 5-HT, and seem less dependent on the isthmic signaling. It is likely that the FGFs from the IsO can only diffuse and activate the genes needed for SA development in the anterior R1.

The caudal spreading of *Otx2* expression is not enough to explain the observed changes in DA and SA populations. Firstly, its expression does
not extend to the whole region where the downregulation of the SA marker genes was observed. Secondly, although Otx2 shows a larger caudal shift in its expression in ventral midbrain, the region where Otx2 spreads is not completely void of SA neurons, although their number is significantly reduced. At the same time, Otx2 expression is scattered and somewhat downregulated in ventral midbrain-R1 region. Based on these observations, it seems that the mixing of MHB region cells, already visible at E10.5, continues during development. At E15.5, the observed cell mixing results to a mosaic midbrain-R1 region where cells of the ventral midbrain, including DA neurons have extensively scattered into ventral R1, but R1 has not changed its fate completely into midbrain. Consequently, there are a mixture of ectopic DA neurons and some SA neurons in the same area. This scattering effect enhances the already impaired development of rostral SA neurons by disrupting the patterning of midbrain-R1 region.

It is interesting that various neuronal populations differ in their requirement for FGF signaling through FGFR1 (see sections 3.1.-3.3). Whereas the midbrain DA neurons shift posteriorly but their numbers are largely unaffected, the rostral SA neurons are greatly reduced in Fgfr1 mutants. Thus, isthmic FGF signaling is required for the proper development of the rostral SA neurons in R1. Our model suggests that high FGF-signaling levels from the IsO prevent neuronal differentiation in the midbrain side near the MHB. When FGF-signaling from IsO is abolished in Fgfr1 mutants, neurogenesis can proceed closer to MHB in the midbrain but is impaired in the ventral R1. This effect is enhanced by the mixing of the cells in ventral midbrain-R1 region which disrupts the patterning of midbrain and R1. Additionally, the slowly proliferating boundary cell population is needed for the positioning and stabilization of the IsO that function by informing the DA and SA progenitor cells in the adjacent brain regions.

4. Identification of a putative target gene of WNT-β-catenin pathway (II)

The first transcript among the downregulated probesets showing an interesting expression pattern was annotated to be a gene called EIG180 (ethanol induced gene product). Further sequence analysis, however, revealed that this transcript is a part of the novel mouse gene located upstream of EIG180 locus on the mouse chromosome 18 (Fig. 19B). This novel mouse gene was found to be orthologous to a human gene called Drapc1 (or Apcdd1), which was earlier showed to be down-regulated in cancer cells by exogenous APC (Takahashi et al., 2002). In addition, they showed that human DRAPC1 is capable to promote growth of cancer cells in vitro and it is a putative target of WNT/β-catenin pathway. The latter notion was further supported by our in situ hybridization analysis. Mouse Drapc1 expression pattern showed striking similarities to active WNT/β-catenin signaling (Maretto et al., 2003) in the developing embryos (Fig. 19A). To support our sequence analysis of the mouse Drapc1 locus and predicted exon structure, we detected similar expression patterns with three individual probes transcribed from different cDNA clones (Fig. 19B). At the midbrain-R1 region, Drapc1 expression was located in the Wnt1- and Otx2-positive cells in the midbrain (Fig. 19D-F). In the Fgfr1 mutants, Drapc1 was
down-regulated at E10.5 and abolished at E11.5 (Fig. 19C).

A blast search against the GenBank EST database (dbest) revealed that Drapc1 was conserved in many vertebrate species as well as in the chordates. To further support our sequence comparisons, we studied chicken Drapc1 expression in early embryos. A marked similarity to the mouse Drapc1 expression pattern was observed (Fig. 20). However, chicken Drapc1 (or Pgo1, NP_001012959 in Genbank) expression was not restricted to the midbrain at the MHB region. This is probably due to the species difference in the regulation of WNT signaling. At this stage (HH 15), however, cell lineage restriction along the anterior and posterior border of the midbrain-R1 boundary might be leaky (Jungbluth et al., 2001). Conservation of the Drapc1 expression pattern between mouse and chicken further suggests DRAPC1 as a putative component of the WNT pathway.

Figure 19. Genomic location of Drapc1 (or Apcdd1) and its gene expression pattern. For the Drapc1 mRNA detection by in situ hybridization we used three EST clones derived from I.M.A.G.E consortium (IMAGE: 5038498, 374528 and 1764103; marked with blue boxes in B). Expression of Drapc1 at E9.0 (A). In Fgfr1 mutants, Drapc1 expression was decreased at E10.5 and E11.5 (C). Localization of Drapc1 in the midbrain overlapping with the Otx2 expression (D-F). A predicted model of Drapc1 transmembrane helices according to TMpred software (G).
5. A slowly proliferating narrow population of cells exists at the boundary between the midbrain and R1 (III,IV).

5.1. Initial changes in gene expression occur close to the midbrain-hindbrain boundary in Fgfr1 mutants (III)

5.1.1. Initial gene expression changes of FGF-FGFR signaling components at E9.5 (III)

A downregulation of Fgf15, Spry1, Spry2, Pea3 and Erm was detected in Fgfr1 mutants at E10.5 by microarray analysis (Table 5). Thus these genes were analyzed at E9.5 with whole-mount in situ hybridization. Interestingly,

Figure 20. Drapc1 expression pattern in chick embryos at 15 and 17 HH stage (Hamburger–Hamilton stage). Whole mount in situ hybridization with Drapc1 antisense (A,C,D) and sense (B) probes. ba1 & 2, branchial arch 1 & 2; IsO, isthmic organizer; mb, midbrain; ov, otic vesicle; psm, presomitic mesoderm; r1, rhombomere 1; sc, spinal cord; te, telencephalon; ZLI, zona limitans intrathalamica.
expression of *Spry1*, *Spry2*, *Pea3* and *Erm* was downregulated only at the narrow boundary region between midbrain and R1 in *Fgfr1* mutants compared to wild-type embryos. Conversely, in *Fgfr1* mutants *Fgf15* expression was expanded to the narrow boundary region where it is not normally detected. These analyses of gene expressions at E9.5 thus suggested that initial changes in the isthmic gene expression in *Fgfr1* mutant embryos occur close to the midbrain-hindbrain boundary.

5.2. Other FGFRs may mediate FGF signaling at a distance from the midbrain-hindbrain boundary (III)

As there are other FGF receptors which can mediate the isthmic FGF signals we compared the expressions of *Fgfr1*, *Fgfr2* and *Fgfr3* in E7.5-E9.5 wild type embryos. In addition, expression of *Fgfr2* and *Fgfr3* was analyzed in E9.5 *Fgfr1* mutants. Based on these analyses *Fgfr1* seems to be the only FGF receptor expressed at the boundary region whereas the expression of *Fgfr2* and *Fgfr3* in the midbrain-hindbrain region was graded gradually diminishing towards the boundary and could not be detected in the cells near the midbrain-hindbrain border by our in situ hybridization analyses. Recently, Blak et al. (2005) reported that *Fgfr2* expression is spanning the midbrain-R1 boundary ventrally at E9.5, which is inconsistent with our data. This, however, would explain why *Spry1*, *Sef*, *Pea3*, *Erm* and possibly *Mkp3* were not completely downregulated in the most ventral midbrain-R1 region in *Fgfr1* mutants. *Fgfr2* and *Fgfr3* expression was not upregulated in *Fgfr1* mutants at E9.5. At E10.5, however, only few *Erm* and *Pea3* positive cells were detected in the midbrain of *Fgfr1* mutants where *Fgfr2* is thought to be expressed at E10.5 (Blak et al., 2005).

5.3. Isthmic constriction fails to develop in the *Fgfr1* CKO mutant embryos (III)

The most obvious morphological landmark at the IsO is a constriction which forms at the boundary between midbrain and R1. In the mouse embryos, the constriction starts to form around E9.25 and it is clearly visible at E10.5. In *Fgfr1* mutants, however, the isthmic constriction was not evidently recognized at E10.5 and was clearly absent at E11.5. These morphological defects further suggested that the cell population at the boundary failed to develop normally and might be required for a stabilization of the IsO.

5.4. Expression of cell cycle regulators at the midbrain-R1 region (I, III, IV)

Several genes responsible for cell cycle regulation were differentially expressed in *Fgfr1* mutants compared with wild-type embryos at E10.5 (Tables 5 and 7). These were validated by in situ hybridization. We found out that *CyclinD1* and *CyclinD2* (or *Ccnd1* and 2) and *CyclinB2* (or *Ccnb1*) were broadly expressed in the dorsal midbrain and hindbrain, but a narrow domain of *CyclinD1*, *CyclinD2* and *CyclinB2* negative cells were detected in the border between midbrain and R1. Microarray screen identified also a negative regulator of the cell cycle, Jumonji (*Jmj* or *Jarid*), which expression was detected as a stripe at the midbrain-hindbrain boundary in wild-type embryos at E9.5 and E10.5. In the *Fgfr1* mutant embryos, *Jmj* was clearly down-regulated at these both stages. Subsequently, we looked at the expression of other cell cycle inhibitors such as *p21*, *p27* and *p57*. Among these only *p21* showed a specific expression at the midbrain-R1 region between E9.0-E11.5. In the wild-
type embryos p21 was expressed as a narrow stripe at the midbrain-hindbrain boundary. In contrast, p21 positive cells were dispersed both in midbrain and R1 in the Fgfr1 embryos at E9.5. Later, p21 expression was completely abolished from Fgfr1 embryos.

5.5. Cell proliferation at the border between midbrain and R1 (III,IV)

5.5.1. The location of p21 expressing cells at the border between midbrain and R1 (III,IV)

Our in situ hybridization analysis revealed the p21-positive cell population within a midbrain-R1 region. Interestingly, p21 expression domain appeared to correspond to the region where CyclinD1/2 and CyclinB1 negative cells were observed. Comparative analysis with Otx2, Wnt1, and Fgf8 determined that these cells were located on both sides of the border between midbrain and R1. The location of p21 expression was further analyzed using immunostaining with anti-p21 antibody and Otx2 in situ hybridization on parallel sections. Consistent with our in situ hybridization analysis, p21 proteins were located on both sides of the midbrain-R1 boundary (Fig. 21). In addition, they were profoundly in the pial side of the neural tube.

Figure 21. Location of the p21 transcripts and proteins in the boundary cells between midbrain and anterior hindbrain (r1) at E11.5. Whole-mount in situ hybridization analysis of p21 expression in wild-type embryos. Parallel coronal sections of E11.5 wild-type embryos were analyzed using anti-p21 antibody (D) and Otx2 radioactive in situ hybridization (E,F). Parasagittal frozen sections immunostained with anti-p21 and anti-PH3 (G-J). The rectangles in C and G show the areas presented in the close-up micrographs D-F and H-J, respectively. Arrowheads show the nuclear p21 expression in the boundary cells (insets in H, I). Arrow in J shows weakly p21-positive cells in the r1, at a distance from the boundary cell population. mb, midbrain; r1, rhombomere 1. Scale bars: 50 μm.
5.5.2. Slowly proliferating cell population at the midbrain-R1 boundary (III,IV)

As several genes regulating the cell cycle showed specific expression patterns at the midbrain-hindbrain boundary we analyzed the cellular proliferation in the midbrain-R1 region. In wild-type embryos (at E10.5), a narrow cell population was found to show a reduced proliferation index assayed by the BrdU incorporation and mitotic cell analyses. Subsequently, this cell population was showed to correlate with the \( p21 \) expression at the border between midbrain and R1. Moreover, we could not detect any \( p21 \)-positive cells which were in S phase of the cell cycle (BrdU analysis) between E10.5 and E11.5 (Fig. 22A-C). Thus, these data suggest that \( p21 \)-expressing cells are a slow-proliferating population which is needed to maintain the coherent boundary between midbrain and R1. To further characterize the role of \( p21 \)-positive cells in the midbrain-R1 boundary region we analyzed the expression of \( p21 \) protein together with KI67 that is expressed at all phases of the cell cycle but is absent from quiescent cells (G0) (Schluter et al., 1993). We could not detect any \( p21 \) proteins overlapping with the KI67-positive cells.

Figure 22. Cell proliferation and \( p21 \) expression in the boundary cells. Micrographs of coronal sections of the midbrain-R1 region at E11.5 immunostained with anti-\( p21 \) and anti-BrdU (A-C). Coronal sections were also immunostained with anti-\( p21 \) and anti-KI67 to detect all cell cycle phases (D-J). DAPI was used to stain the nuclei. BrdU injection was given 1 hour before the mice were sacrificed. Dorsal (A-C,D,E, H-J) and ventral (F,G) regions are shown. Orange arrowheads indicate cells expressing both \( p21 \) and KI67 in the midbrain (E). White arrowheads point to the boundary between midbrain and R1. mb, midbrain; r1, rhombomere 1. Scale bars: 50 \( \mu \)m.
at the vicinity of the boundary cells (Fig. 22E,G,I-J). However, p21 and KI67 were co-expressed in the same cells further away from the boundary cells (IV, Fig. 22E, orange arrowheads).

The narrow boundary cell population is dependent on *Fgfr1* signaling and may prevent cell mixing between midbrain and R1. In *Fgfr1* mutants, loss of p21 expression might interrupt the normal cell cycle kinetics leading into cell sorting and/or cell fate changes at the midbrain-R1 boundary region (see Fig. 24 below). Consistent with this, both *Wnt1* and *Otx2* were ectopically expressed in the R1 region in *Fgfr1* mutants at E10.5. Because we also detected downregulation of cell adhesion molecules (e.g. *Pb-cadherin* and *Ob-cadherin*, Fig. 23), a decrease in the p21 expression level might be due to the loss of these cells. In addition, *Fgfr1* is not absolutely required for the development of the boundary cells as scattered p21 expressing cells were observed at E9.5 in *Fgfr1* mutants.

### 5.5.3. The contribution of the boundary cell population to the midbrain-R1 development (IV)

The cell lineage properties of the boundary cell population were studied with a mouse line expressing the Cre-recombinase in a boundary cell –specific fashion (designated MHB-Cre mouse line). We detected a marked similarity between the MHB-Cre activity and the p21-expressing boundary cells between the E10.5-E11.5.

![Figure 23. The loss of cell adhesion properties in the Fgfr1 mutant.](image)

In addition to the *Pb-cadherin* (Trokoovic et al., 2003), we also detected the downregulation of *Ob-cadherin* at the midbrain-R1 region in *Fgfr1* mutant embryos at E10.5 (B).

More detailed analysis showed that a subset of recombined cells (*MHB-Cre/+; R26R/+* embryos) overlapped with the p21 expression in the most posterior midbrain and the most anterior R1. As we could not detect any p21-expressing cells that were in S phase, this observation suggests that boundary cells may represent a slowly proliferating stem cell population. Additional hypothesis suggests that the p21-expressing cells might be differentiating within the Cre-expressing domain between E10.5-E11.5.

Fate mapping data of *MHB-Cre/+; R26R/+* embryos revealed that the midbrain-R1 boundary cells marked by MHB-Cre contributed to the midbrain and R1 derivatives such as inferior colliculi and cerebellar vermis, respectively. Interestingly, the majority of recombined cells remained near the border between midbrain and R1, further suggesting tightly controlled lineage restriction between these brain compartments.

Our results suggest that p21-expressing boundary cells posses a decreased proliferation potential and would require re-activation of self-
renewal pathways. Decrease of *Cyclin D1/2*, by limiting the proliferation of early boundary cells, would reduce the number of cells arising from the early border between midbrain and R1. Although p21 expressing nuclei are located at the basal side there is fewer cells which are entering the cell cycle. In the midbrain-R1 region a domain of reduced cell proliferation has been reported earlier in E14 rat (Altman and Bayer 1997) and in E12.5 mouse (Li et al., 2002). In these studies the region of slowly proliferating cells was co-localized with the broad *Fgf8* (100 μm) expression domain in the R1, whereas in our analyses the reduced cell proliferation rates and p21 (50 μm) expression were detected only in the most anterior part of the *Fgf8* expression domain (app. 25% of it) and in the most posterior midbrain.

**Figure 24. Maintenance of compartments between midbrain and R1 is dependent on *Fgfr1* signaling.** In a cell sorting-based model, cells cannot move to the other side of the boundary (Aa, a cell sorting-based mechanism) whereas in a cell fate-based mechanism cells regulate their gene expression to match that of their neighbors (Ab). Different cell adhesion properties of midbrain and R1 cells (e.g. *Pb-cadherin*) and our data of existence on boundary cell population favor the presence of cell sorting at the IsO. In wild-type embryos this sorting mechanism leads to a sharp *Wnt1/Fgf8* expression boundary (A; E10.5). Conversely, in *Fgfr1* mutants (Mut) disruption of a coherent signaling from the IsO and subsequent loss of the cell sorting mechanism leads to a cell mixing at the border between midbrain and R1 (B; E10.5). White arrowheads point to the cell mixing in the R1.
CONCLUSIONS

The Iso provides as one of the best models to study the cellular mechanism of the regionalization and differentiation of the brain along the AP axis. Two secreted factors, FGF8 and WNT1, are required for the Iso activity and the development of the midbrain and cerebellum.

In this study we have studied the role of FGFR1 in the development of midbrain-R1 region. We compared the gene-expression profiles of wild-type and Fgfr1 CKO mutant embryos in order to discover new molecules regulating the Iso activity and development of this brain region.

In this work, we have characterized mouse genes that are involved in the FGF-FGFR1 signal transduction in the midbrain-R1 region. Our gene-expression analyses showed that a number of these molecules are regulated by FGFR1 signal transduction pathway and they show specific expression at the Iso. These molecules are modulating the FGFR signaling either positively (CNPY1, FLRT3) or negatively (MKP3, SEF).

The present study suggests that the mouse CNPY1 is a novel FGFR1 pathway component which is essential for the FGF signal transduction at the Iso. It has been hypothesized that CNPY1 activity is needed to attenuate the FGFR signaling through physical interaction with the lipid rafts on the plasma membrane (Hirate and Okamoto 2006). This assumption provides an interesting mechanism how the activity of broadly expressed FGFR1 is modulated in the vicinity of the Iso.

In addition to components of FGFR signal transduction, our gene-expression profiling revealed a transcript, Drapc1, which was found to be a novel component of the WNT signaling. Although, its expression at the MHB is striking, the actual role of DRAPC1 in the midbrain or hindbrain development needs to be resolved.

Prior to this study the role FGFR1 in the early development of neuronal populations such as DA and SA neurons was not addressed. The data from microarray screen showed that a number of genes regulating the neuronal differentiation were up-regulated in the Fgfr1 CKO mutants. Further studies with conditional Fgfr1 mutants provided in vivo evidence for the involvement of FGF signaling in the development of DA and SA cell-types characteristic for the ventral midbrain and R1, respectively. In the developing midbrain DA neuron population, we suggest that FGF signaling is required for active proliferation of the early precursor cells rather than the neural differentiation process itself. FGF signaling likely regulates the development of other neuronal precursor pools in the midbrain-R1 region in a similar fashion. However, due to redundancy of different FGFRs further analyses of FGFR1-3 compound mutants are needed to clarify the role of FGFR signaling in the neuronal development.

About fifteen years ago the existence and importance of compartment boundaries was discovered in the embryonic rhombomeres (Fraser et al. 1990). It was postulated that compartment boundaries in the developing CNS have a dual function during development and serve a similar function with the Drosophila embryonic structures such as imaginal discs. Firstly, neuroepithelial compartment boundaries are preventing the intermingling of cells that are fated to contribute to different parts of the embryo (e.g. midbrain vs.
R1). Secondly, compartment boundaries are providing positional information to adjacent cell populations. In addition to the compartmentalization of rhombomeres, it was also shown that the positional identity of each rhombomere is defined by the combinatorial expression of homeotic selector genes such as the *Hox* genes. Thus, after the initial studies of rhombomeric boundaries there has been continuous search for compartments and these so-called selector genes in other regions of the developing brain as well.

Before this study was initiated, it was not clear whether the midbrain-R1 compartment boundary really exists. For example, Jungbluth et al. (2001) proposed that midbrain-R1 boundary does not represent a compartment boundary in the chick embryos (HH 11 stage). The loss of isthmic constriction in the *Fgfr1* mutants by E10.5 together with the deregulation of the cell cycle regulators prompted us to study the cellular characteristics behind this morphological landmark (see Fig. 24). Subsequent characterization of the cell proliferation at the boundary between midbrain and R1 identified a slowly proliferating cell population (*p21*-expressing cells) that may contribute to compartmentalization of this brain region. Furthermore, in the present study, we present that one of the main aspect of the boundary between midbrain and R1 is to position and stabilize the local signaling centre, the IsO.

To support our hypothesis of compartmentalization at the midbrain-R1 region Brand and co-workers (Langenberg and Brand, 2005) have reported recently a state-of-the-art time-lapse study, in which they mapped the fates of hundreds of cells in the developing zebrafish midbrain-R1 region. They proposed that cells do not cross the border between midbrain and R1 during the zebrafish development. In mice, the existence of cell lineage restriction at the midbrain-R1 boundary was supported by a study that used genetic fate mapping with an inducible transgene (Zervas et al., 2004). Both these analyses clearly showed the presence of cell lineage restriction boundary between the midbrain and R1, but it was proposed that in the mouse the cell lineage restriction is set up at different phases in ventral and dorsal midbrain-R1 domains (Zervas et al., 2004). Importantly, our analysis with the cells marked by *MHB-Cre* further suggests limited cell mixing within these brain compartments.

In addition, the role of slowly proliferating boundary cells between midbrain and R1 has been recently confirmed by others. Baek et al. (2006) showed that a high expression levels of transcription factor *Hes1* in the border between midbrain and R1 is needed for the coherent boundary formation. This narrow expression domain of high *Hes1* expression level correlates with the *p21*-expressing cells.
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