Molecular characterization of the kinocilia-specific wdr16 promoter

"Molekulare Charakterisierung des kinocilien-spezifischen wdr16-Promoters"

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Abbreviations

Ad	Adenovirus
AP	Alkaline phosphatase
APS	Ammonium peroxodisulfate
bp	base pairs
BS	Binding sites
CAR	Coxsackie–adenovirus receptor
CM(s)	Conserved motif(s)
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
CTRL	Control
d	day(s)
ddH20	Double deionized water
DIV	Day(s) in vitro
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNAH9	Dynein, axonemal, heavy chain 9
DNAI1	Dynein, axonemal, intermediate chain 1
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor 1 alpha
EGFP	Enhanced green fluorescent protein
EMSA	Electrophoretic mobility shift assay
EPC	Ependymal primary culture(s)
FFL	Firefly luciferase

FGF	Fibroblast growth factor(s)
Fig.	Figure
FKH-2	Forkhead 2
FOXJ1	Forkhead box J1
GFP	Green fluorescent protein
GSL	Gaussia luciferase
HBS	HEPES-buffered saline
HEK	Human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV-1	Human immunodeficiency virus type 1
HMG	High-mobility group
HNF1β	Hepatocyte nuclear factor 1β
HSV-1	Herpes simplex virus type 1
LB	Luria bertani
LV	Lentiviral vector
MEM	Minimal Essential Medium
MOI	Multiplicity of infection
NCBI	National Center for Biotechnology Information, Bethesda, USA
OD	Optical density (absorbance/extinction)
PAGE	Polyacrylamide gel electrophoresis
PA	Polyacrylamide
PBS	Phosphate-buffered saline
Pkhd1	Polycystic kidney and hepatic disease 1
PMSF	Phenylmethanesulfonylfluoride
PPT	Polypurine tract
PS	Penicillin/streptomycin
PWM	Position weight matrix
RFX	Regulatory factor X

rpm	Revolutions per minute
RSHL2	Radial spoke-head-like 2
RSPH1	Radial spoke head 1
RT	1. Reverse transcriptase
	2. Reverse transcription
	3. Room temperature
SCO	Subcommissural organ
SEAP	Secreted alkaline phosphatase
shRNA	Short hairpin RNA
SOC	Superoptimal broth for catabolite repression (medium for bacterial liquid culture)
SP1	Specificity protein 1
SPAG6	Sperm-associated antigen 6
ssRNA	Single stranded RNA
stx8	Syntaxin 8
SV40	Simian virus 40
SVZ	Subventricular zone
TEMED	N,N,N',N'-tetramethylethylenediamine
TFBS	Transcription factor binding site
TF	Transcription factor(s)
TRIS	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
YPD	Yeast peptone dextrose

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1. Introduction

1.1. The ventricular system

The structures of the ventricular system are embryologically derived from the centre of the neural tube. The ventricular system of the brain consists of four irregularly shaped cavities called ventricles:

- Two lateral ventricles (right and left).
- The midline third and fourth ventricles connected by the cerebral aqueduct.

Cerebrospinal fluid (CSF), largely secreted by the choroid plexus of the ventricles, fills these brain cavities and the subarachnoid space of the brain and spinal cord. The lateral ventricles, i.e., the first and second ventricles, are the largest cavities of the ventricular system and occupy large volumes of the cerebral hemispheres. The third ventricle, a slit-like cavity between the right and left halves of the diencephalon is continuous posteroinferiorly with the cerebral aqueduct, a narrow channel in the midbrain connecting the third and fourth ventricles. The third ventricle communicates with the lateral ventricles through a small opening at its anterior end called the interventricular foramen. The pyramid-shaped fourth ventricle in the posterior part of the pons and medulla extends inferoposteriorly. Inferiorly it tapers to a narrow channel that continues into the cervical region of the spinal cord as the central canal.

1.2. Cerebrospinal fluid and its circulation

The CSF is a major part of the extracellular fluid of the central nervous system. The CSF fills the ventricles of the brain, the subarachnoid space and the spinal canal. The CSF is produced by the choroid plexus in the two lateral as well as the third and fourth ventricles. The CSF is produced constantly, and in humans the total volume is approximately 140 ml and is replaced about four times a day. Thus, the total amount of CSF produced in one day is about 600 ml.

The CSF is separated from neuronal tissue by the ependyma and the pia which covers the external surface of the brain. The CSF has many important functions. CSF serves as a drainage pathway for the brain, by providing a "sink" into which products of metabolism or synaptic activity are diluted and subsequently removed (Segal, 1993). The CSF may also be an important passage by which some nutrients reach the CNS (Johanson et al., 1999). CSF may also acts as a route of communication within the CNS by carrying transmitters and hormones between different areas of the brain (Johanson et al., 1999)

CSF leaves the lateral ventricles through the interventricular foramen of Monro and enters the third ventricle. From there CSF passes through the cerebral aqueduct into the fourth ventricle. From the fourth ventricle, it can flow into the central canal of the spinal cord or into the cisterns of the subarachnoid space through three small foramina: the central foramen of Magendie and the two lateral foramina of Luschka. CSF also flows into the extensions of the subarachnoid space around the cranial nerves. The fluid then passes around the superior sagittal sinus to be reabsorbed through the arachnoid villi into the venous system (Nagra et al., 2006). CSF within the spinal cord can flow all the way down to the lumbar cistern at the end of the cord around the cauda equina. The directional beating of the ependymal cilia may play a role in the circulation of CSF (Davson and Segal, 1995), as evidenced by the development of hydrocephalus in small animals suffering from primary ciliary dyskinesia (Greenstone et al., 1984; Afzelius, 1985)

1.3. Ependymal cells

The lining of the cerebral ventricles and central canal of the spinal cord is similar to most epithelia — in both form and construction. The character of the epithelial lining was first documented by Purkinje (1836). Investigators found that the ependyma was of heterogeneous composition (Agduhr, 1932), in particular that some cells had basal processes that extended into the subjacent neuropil (Wislocki, 1932). Horstmann (1954) first used the descriptive term tanycyte to such elongated ependymal cells seen in selachians (any fish belonging to the subclass *Selachii*) with gold sublimate staining. In humans, the ependymal lining is complete by approximately 26-28 weeks of gestation. Mature ependymal cells are morphologically characterized by a cuboidal to columnar shape,

a more or less round nucleus with fine-stippled chromatin pattern, and an inconspicuous nucleolus. Microvilli cover their surface, and most of the cells have a central cluster of long cilia. A network of supraependymal axons is located on the surface of the ependymal layer (Del Bigio, 2010).

1.3.1. Ependymal development and neurogenesis

A ciliated ependymal cell does not exist along the immature neural tube. As the ventricular zone and radial glial cells regress, the timing of which is region-specific and species-specific, the central canal and the ventricles become gradually covered by ciliated ependyma (Del Bigio, 2010). Ependymal cells may represent a terminally differentiated offspring of the proliferating ventricular zone (Tramontin et al., 2003) or a more gradual terminal transformation of radial glial cells (Webster and Astrom, 2009). In the mouse spinal cord ependymal cells have been reported to be derived from Nkx6.1 expressing ventral neuroepithelial cells (Fu et al., 2003). On the other hand, in mouse forebrain ependymal cells appear to be derived from radial glia (Spassky et al., 2005). In adult mice, ependymal disruption shows that subventricular zone cells can create replacement of ependymal cells (Kuo et al., 2006). It is shown that morphologically distinct ependymal cells in human can develop regionally from the undifferentiated neuroepithelium along a caudal-to-rostral gradient (Sarnat, 1992). During the fetal period, basal processes in most ependyma can extend into the parenchyma but are distinguishable from the radial glia (Sarnat, 1992).

The more recent research on ependymal cells suggest the possibility that the EPC can de-differentiate into stem cells (Coskun et al., 2008). The presence of a subpopulation of CD133 in ependymal cells may indicate an additional, quiescent stem cell population in the mammalian forebrain (Pfenninger et al., 2007). High resolution analysis of ependymal cell proliferation in rat brain following injury by the toxin 6-hydroxydopamine indicates that ependymal cells can divide asymmetrically and transfer progeny into the subventricular zone (Gleason et al., 2008; Del Bigio, 2010).

Suppression of the constitutive expression of the notch signaling pathway allows ependymal cells in rats with cerebral ischemia (Carlen et al., 2009) to reacquire a radial glia phenotype

and to subsequently generate neuroblasts (Zhang et al., 2007). In lower vertebrates such as lamprey, fish and salamanders, the cells lining the ventricle of the spinal cord, and perhaps the brain, persist in the radial glia form and can contribute to regeneration and neurogenesis even in adults (Endo et al., 2007; Tanaka and Ferretti, 2009). New evidence suggests that a small cluster of EPC in the dorsal pole of the central canal of the mature mouse spinal cord, distinct from subventrilcular zone cells, may also have stem cell potential following spinal cord injury (Hamilton et al., 2009; Moreno-Manzano et al., 2009).

1.3.2. Functions of mature ependymal cells

The mature ependymal cell is capable of only limited proliferation and repair. In pathological situations, such as viral Infection and hydrocephalus, destruction was the main outcome (Bruni, 1998). In 1992, Sarnat demonstrated the secretory functions of fetal ependymal cells in detail but wrote that the ‘‘ependyma of the adult brain may be little more than a decorative lining of the ventricular system’’ (Sarnat, 1992). The expression of the extracellular matrix proteins which assist axon guidance was found in basal surfaces of ependymal cells, at least in lower vertebrates. Sarnat speculated that the transformation of radial glial cells and the subsequent development of a variety of brain malformations might be influenced by secreted molecules. Finally, he alluded to a transport role of ependymal cells for moving small molecules between CSF and brain. The general inability of ependymal cells to proliferate, the development of reactive astroglial changes and buried clusters of ependymal cells at sites of erosion, and viral infection of the ependyma could result in hydrocephalus (Sarnat, 1995). Mature mammalian ependymal cells possess the structural and enzymatic characteristics essential for scavenging and detoxifying a wide variety of substances in the CSF, and the potential of forming a metabolic barrier at the brain–CSF interface. The presence of motile cilia, microvilli, and adherence junctions at the apical surfaces is of importance for these roles (Del Bigio, 2010).

The presence of gap junctions plays a crucial role in coordinating the activities of adjacent cells (Del Bigio, 1995). The ependymal cells can upregulate the protective proteins that might prevent the entry of harmful metabolites from CSF back into the brain tissue (Del Bigio, 2002). The association between ciliated ependymal cells and pluripotent cells has also

led to investigation of ependyma as a modulator of stem cell populations. In adult cattle, ependymal cells maintain basal processes in regions of the frontal horns where subventricular zone cells are most abundant (Rodriguez-Perez et al., 2003). Some have proposed that ependymal cells organize the subventricular zone through production of specific extracellular matrix and adhesion molecules (Hauwel et al., 2005a) or through release of other modulators (Sarnat, 1992). Ependymal cells may contribute to the trophic function through the production of growth factors. Fibroblast growth factors (FGF) are among the most important growth factors in brain development (Iwata and Hevner, 2009). FGF2 (also known as basic FGF) has repeatedly been described in mature ependymal cells of rodents (Cuevas and Gimenez-Gallego, 2000; Fuxe et al., 1996). Observations of an increased FGF2 level following ischemia suggest a role in trophic support of adjacent cells (Hayashi et al., 1998). Vascular endothelial growth factor (VEGF) is expressed by human ependymal cells between 22 and 40 weeks of gestation (Arai et al., 1998). It may have autocrine and paracrine functions. VEGF plays a role in ependymal stability; the disappearance of microvilli was seen when action of VEGF was prevented in mice (Maharaj et al., 2008). In dystrophin-deficient mdx mice, VEGF is upregulated in the ependyma (Nico et al., 2006, 2009) and in rat ependyma following ischemia (Wang et al., 2008). Endothelial growth associated Ang-1 (Horton et al., 2010), Tie-2 (Nourhaghighi et al., 2003), and Flt-1 (Tonchev et al., 2007), which are present in rat ependymal cells, appear to be involved in the autocrine function.

In addition to the potential of ependymal cells to support cell populations in the adjacent subventricular zone through trophic factors, emerging evidence shows the potential for metabolic regulation of adjacent cells. Glucose uptake by ependymal cells from CSF can occur through facilitative glucose carriers; ependymal cells are reported to possess GLUT1 (Silva-Alvarez et al., 2005), GLUT2, GLUT3 (Yu et al., 1995), and GLUT4 (Kobayashi et al., 1996). The uptake of glucose into cultured ependymal cells can be stimulated by insulin and insulin-like growth factor (Verleysdonk et al., 2004). In ependymal cells glucose can be converted to glycogen (Prothmann et al., 2001), which can then be mobilized by noradrenaline and serotonin, suggesting that these cells maintain glycogen as a regulated energy store (Verleysdonk et al., 2005).

1.3.3. Infection of ependymal cells by viruses

Ependymal cells are susceptible to a variety of common viruses including varicella zoster (Kleinschmidt-DeMasters et al., 1996, 2001), pseudorabies (Chen et al., 1999), Hendra virus (Wong et al., 2009), cytomegalovirus (Fritschy et al., 1996; Han et al., 2007; Reuter et al., 2004), Dengue virus (An et al., 2003), Semliki Forest virus (Fragkoudis et al., 2007), human T cell leukemia virus type I (Coscoy et al.,1996), murine lymphocytic choriomeningitis virus (LCMV) (Kappes et al., 2000), Herpes simplex virus type 1 (HSV-1; Kesari et al., 1998), measles virus (Ludlow et al., 2008), mumps virus (Takano et al.,1999), vesicular stomatitis virus (VSV; Plakhov et al., 1995), the neurovirulent A/WSN/33 (H1N1; WSN) strain of influenza A virus (Takahashi et al., 1995), Nipah virus (Torres-Velez et al., 2008), and Borna disease virus (Werner-Keiss et al., 2008). The coxsackie–adenovirus receptor (CAR) is a transmembrane protein expressed in the developing central nervous system but restricted to ependymal cells in the adult brain (Hauwel et al., 2005b). Human ependymal cells also express the gene for measles virus receptors CD46 (membrane cofactor protein), an expression they share with many other cell types (McQuaid and Cosby, 2002).

The viral receptors in the ependymal cells facilitates the use for genetic manipulation experiments in which a gene can be linked to a viral vector and injected into the ventricle where it is taken up by mature ependymal cells (Del Bigio, 2010). Among these are the adeno-associated virus vectors (Davidson et al., 2000; Liu et al., 2005; Tenenbaum et al., 2004; Wang et al., 2003), Sendai virus vector (Inoue et al., 2004), herpes simplex type 1 vectors (Furlan et al., 1998), and lentivirus vectors (Dolcetta et al., 2006; Geraerts et al., 2006; Watson et al., 2005),

1.4. Lentiviral–vector mediated gene transfer

The use of lentiviral vectors as a tool for gene delivery has attracted significant interest and has developed as a promising tool for transgenic application. Indeed, retroviral vectors have the same ability as lentiviral vectors to stably integrate into the target cell genome. However, the property of lentiviral vectors of offering efficient transduction of target genes into nondividing cells makes them to be in obvious advantage for the consideration as a tool

for gene transfer (Mátrai et al., 2010). Another advantage of using lentiviral vectors is their capability to accommodate larger transgenes (up to approximately 10 kb) than retroviral vectors (De Meyer et al., 2006).

In general, lentivector particles are generated by the co-transfection of three plasmids in human embryonic kidney (HEK) 293T cells (Naldini et al., 1996), a packaging plasmid, a transfer plasmid, and an envelope-encoding plasmid. The tropism of lentiviral vectors is determined by their viral envelope glycoproteins. The interaction of the viral envelope glycoprotein with their receptors triggers the fusion of the viral envelope with the plasma membrane of the target cell. The production of lentiviral vector preparations of high titer was not achievable due to the restricted tropism in wild-type HIV glycoproteins. Therefore, heterologous glycoproteins were used for the production of lentiviral vectors, a process termed ‘‘pseudotyping’’. Envelope glycoprotein of vesicular stomatitis virus (VSV G) is often used for pseudotyping. The transfer plasmid contains an expression cassette and the genes of HIV cis-acting factors, proteins necessary for packaging, reverse transcription, and integration (Escors and Breckpot, 2010). They can be modified to augment lentiviral vector performance and safety. Nuclear import of the transfer construct was improved by including the polypurine tract and its central termination sequence, together forming a triple helix, resulting in higher vector titers and enhanced transgene expression (Sirven et al., 2000). Thus, the packaging plasmid contains the coding sequences for gag and pol, the viral regulatory genes tat and rev, and the accessory genes vif, vpr and vpu. Gag encodes the structural proteins, whereas the pol gene encodes the enzymes that accompany the single stranded RNA. Of these, reverse transcriptase carries out reverse transcription of the viral RNA to DNA, integrase catalyses the integration of the proviral DNA into the host genome, and protease is involved in gag-pol cleavage and virion maturation (Katz and Skalka, 1994).

1.4.1. Lentivirus mediated gene transfer in ependymal primary cultures

Ependymal primary cultures (EPCs) are an established model for studying ependymal cell biochemistry and the biology of kinocilia-bearing cells (Verleysdonk, 2006a); Kowtharapu et al., 2009). However, the difficulty in causing them to express transgenes at high efficiency

has been an important drawback of the system. Indeed plasmid-based transfection attempts remain at efficiency below 1% and fail to elicit reporter gene expression, namely green fluorescent protein (GFP) synthesis, in any of the kinocilia-bearing cells of the cultures. Human immunodeficiency virus pseudotyped with the vesicular stomatitis virus envelope glycoprotein (HIV/VSV-G) and encoding GFP under the control of the ubiquitously recognised promoter of elongation factor 1 alpha (EF1alpha) also does not cause transgene expression in the kinocilia-bearing cells of an EPC when applied at multiplicities of infection (MOI) of up to 40, and destroys the culture when the MOI is increased further. In contrast, HIV/VSV-G encoding GFP under the control of a promoter specifically active in kinocilia-bearing cells leads to transgene expression in up to 79% of the kinociliated cells of an EPC when applied at an MOI of 20 (Kowtharapu et al., 2009). This has permitted the characterisation of the promoter for the gene specifically transcribed in kinocilia-bearing cells, wdr16.

1.5. Different types of cilia

Cilia are classified on the basis of their axonemal architecture and their motile properties. The axoneme of primary cilia consists of 9 doublets of peripheral microtubules with no central pair (9+0 axoneme). Primary cilia are generally non-motile with an exception of the primary cilia in the mouse embryonic node. However, most of the motile cilia are composed of an axoneme that comprises 9 outer doublets of microtubules and an additional central pair (9+2). There are specific structures associated with the 9+2 axoneme, such as radial spokes. Outer and inner dynein arms ensure the motility of cilia or flagella and can be found on 9+0 (embryonic node) or 9+2 axonemes. A variety of exceptions can be found to this stereotyped 9+0 and 9+2 axonemal architecture (Mencarelli et al., 2008).

1.5.1. Ependymal cilia

The most distinguished structural feature of ependymal cells is their apical cluster of cilia. It has been shown that ependymal cilia beat in a synchronized manner, sweeping tracers along the same direction as CSF flow (Del Bigio, 2010). The communication through gap junctions or through innervations facilitates the synchronized beating of the cilia. It has

been shown that cilia serve to clear mucus and inhaled particles in the airways (Mall, 2008). The role of cilia in the CSF is not clear, although it has been postulated that they stir CSF to facilitate distribution of molecules (Nelson and Wright, 1974). Recently, it has been demonstrated that ependymal cilia may play a role in creating concentration gradients of guidance molecules in CSF that serve to direct neuroblast migration from the lateral ventricle wall into the olfactory bulb (Sawamoto et al., 2006). Atrophy of ependymal cilia occurs prior to loss of ependymal cells in hydrocephalus induced by mechanical obstruction to CSF flow (Del Bigio, 1993). Knockdown of the kinocilia specific gene wdr16 in zebrafish leads to severe hydrocephalus with apparently intact ciliary movement and no visible alterations of the ependymal layer (Hirschner et al., 2007).

1.5.2. Intraflagellar transport

Intraflagellar transport was first identified in *Chlamydomonas* as a bidirectional transport of proteins along the axoneme (Kozminski et al., 1993) and has been demonstrated to be conserved and essential for the assembly of cilia in almost all organisms, with the exception of the *Plasmodium falciparum* (Han et al., 2003) and Drosophila flagellae (Briggs et al., 2004). Anterograde transport is driven by Kinesin II, whereas retrograde transport is ensured by the cytoplasmic dynein complex (Thomas et al., 2010).

1.6. wdr16 promoter

wdr16 is a gene the product of which is only present in kinocilia-bearing, but absent from all other cells, even from those with a primary cilium (Hirschner et al., 2007). The orthologues of the wdr16 gene are especially suited as a paradigm for the analysis of kinocilia-specific promoters. These are defined here as promoters which are only active in kinocilia-bearing cells. They exhibit a special form of shared synteny, in which the wdr16 gene lies in “head-to-head” orientation to the syntaxin 8 (stx8) gene with less than 1 kb separating the respective transcription start sites in many species (Hirschner et al., 2007).

1.7. Transcriptional control of ciliary gene expression

Historically, transcriptional regulation of ciliary gene expression was first documented in C*hlamydomonas* and sea urchins (Rosenbaum et al., 1969), in which deflagellation or deciliation can be induced by different kinds of chemical or mechanical stress. In *Chlamydomonas*, it takes approximately 90 min for regrowth of the flagella which starts immediately after experimentally induced deflagellation (Rosenbaum et al., 1969). The flagella can be assembled by using a preexisting pool of proteins which is sufficient to reconstitute a half-length flagellum. However, to have complete ciliary growth, the transcription of flagella genes must be switched on (Lefebvre et al., 1978, 1980; Silflow et al., 1982). It has been shown that accumulation of α- and β-tubulin mRNA, after the induction of deflagellation, due to increased transcription which results in the appearance of new mRNAs (Keller et al., 1984). More recently, microarray hybridization revealed significant variations of levels of mRNA derived from various ciliary genes during *Chlamydomonas* flagellar resorption or reassembly (Pazour et al., 2005), but changes in either mRNA synthesis or stability could account for these variations (Stolc et al., 2005; Chamberlain et al., 2008; Thomas et al., 2010).

Although the transcriptional control of ciliary gene expression in experimentally induced deflagellation of *Chlamydomonas* has been studied, the mechanisms underlying this regulation are not yet known in this organism (Thomas et al., 2010). Many studies have been aimed mainly at the identification of regulatory sequences in the promoters that control the expression of tubulin genes. Nevertheless, a consensus sequence on tubulin gene promoters has not become known and no specific molecular players have been isolated so far. However, searches for proteins that are specifically induced (at either the transcriptional or the translational level) during flagellar regeneration in *Chlamydomonas* have shown to be highly instructive in finding many ciliary structural components as well as regulators of their assembly (Pazour et al., 2005; Stolc et al., 2005) by identifying the key players controlling the transcriptional regulation of cilia-specific genes. In this respect two major families of proteins have been identified: the TF forkhead box J1 (FOXJ1) and

regulatory factor X (RFX). More recently, an S-SOX5 SRY-related (term used by the authors Kiselak et al., 2010) HMG TF was identified in the transcriptional regulation of sperm-associated antigen 6 (SPAG6) (Kiselak et al., 2010). It has also been reported that the up-regulation of Sox11b mRNA after spinal cord injury is mainly located in ependymal cells lining the central canal and in newly differentiating neuronal precursors or immature neurons (Guo et al., 2010).

1.7.1. FOXJ1 transcription factors

FOXJ1 is a member of the forkhead/winged-helix family of TF, and orthologues of FOXJ1 have been found in vertebrates and many invertebrates, including sea urchins and the planarian model *Shmidtea mediterrannea,* an emerging tool for studying the genesis of motile cilia. In ecdyzozoa such as *Caenorhabditis elegans* and *Drosophila melanogaster* no orthologues of FOXJ1 have been identified (Mazet et al., 2003). Several pieces of evidence support the hypothesis that FOXJ1 is essential for the generation of motile cilia. FOXJ*1* is expressed in several mammalian tissues equipped with motile cilia (Hackett et al., 1995; Lim et al., 1997; Brody et al., 2000). For example, expression of Foxj1 in the lung is correlated with the growth of motile cilia (Blatt et al., 1999), but not in the case of non-motile primary cilia (Jain et al., 2010). Hydrocephalus and left–right asymmetry defects, two phenotypes known to result from defective motile cilia were found in FOXJ1 knock out mice. Indeed, motile 9+2 cilia were absent in the nasal epithelium and ventricular cells of FOXJ1 knock out mice (Jacquet et al., 2009). In zebrafish and *Xenopus* FOXJ1 plays a role as a master regulator of the program for motile cilia. The ectopic expression of FOXJ1 in the zebrafish or in the *Xenopus* epidermis was enough to induce growth of motile cilia in various tissues of the zebrafish or in *Xenopus* epidermis (Stubbs et al., 2008; Yu et al., 2008; Thomas et al., 2010).

Recently, several FOXJ1 target genes have been identified in *Xenopus* (Stubbs et al., 2008), zebrafish (Yu et al., 2008) and mice (Jacquet et al., 2009), out of which many are shown to be involved in ciliary motility. Approximately hundred genes were upregulated (Gherman et al., 2006) more than ten fold by the FOXJ1 overexpression in the epidermis *Xenopus* (Inglis et al., 2006), among which one third are found in databases of ciliary proteins (Arnaiz et al.,

2009). Many of these genes encode proteins found exclusively in motile axonemal structures such as subunits of axonemal dyneins DNAH8 and DNAI1 (dynein, axonemal, intermediate chain 1)], [DNAH9 (dynein, axonemal, heavy chain 9), Wdr16 (WD repeat domain 16) (Hirschner et al., 2007), SPAG6 (sperm-associated antigen6) and various radial spoke proteins [RSHL2 (radial spoke-head-like 2), RSHL3 and RSPH1 (radial spoke head 1)]. These findings show that FOXJ1 proteins play an important role in regulating genes essential for the function of motile ciliary genes

1.7.2. RFX family of transcription factors

Another major type of TF which has been demonstrated to be of importance in the regulation of ciliogenesis in *C. elegans*, *Drosophila* and vertebrates is the RFX proteins. These were first found by their ability to bind to the so-called X-box motif that is conserved in major histocompatibility class II gene promoters (Reith et al., 1990). RFX proteins share a highly conserved DNA-binding domain which belongs to the winged-helix family of TF (Gajiwala and Burley 2000). One characteristic feature of the RFX subgroup of winged-helix proteins is that they use their so-called recognition motif to bind to the minor groove of DNA (Gajiwala et al., 2000). The involvement of members of RFX proteins in the control of ciliogenesis was first found on *C. elegans*. Swoboda et al. (2000) demonstrated that the TF DAF-19 is necessary for sensory cilia assembly in *C. elegans*. This finding led the way for several studies in which the X-box motif was used to identify novel genes involved in ciliogenesis in *C. elegans* and in other organisms (Haycraft et al., 2001, 2003; Schafer et al., 2003; Avidor-Reiss et al., 2004; Blacque et al., 2005; Efimenko et al., 2005; Chen et al., 2006; Laurençon et al., 2007). RFX transcription factors have been shown to regulate a particular subset of ciliary proteins which are involved in molecular transport that is important for cilia assembly and function (Thomas et al., 2010).

1.7.3. SOX transcription factors

The sox family consists of 20 genes, which encode TF with a high-mobility-group (HMG) box DNA binding domain. This domain is similar to that of the sex-determining region (Sry) protein (Degnan et al., 2009; Phochanukul and Russel, 2010). On the basis of phylogenetic

analysis of their HMG domains, SOX genes are divided into ten groups, A to J (Chew and Gallo, 2009). Many genes within each subgroup also share conserved regions outside the HMG domain. The binding of SOX proteins to specific DNA sequences can activate or repress target genes (Reiprich et al., 2010; Hernandez-Hernandez et al., 2009). It has been shown that SOX proteins are involved in the regulation of diverse developmental processes such as development of lens (Donner et al., 2007), muscle (Savage et al., 2009), blood vessel (Cermenati et al., 2008), hair follicle (Kanai-Azuma et al., 2002), gut (Christiano, 2008), B cells (Schilham et al., 1996), and cartilage (Haag et al., 2008; Bonnelye et al., 2007). SOX genes are expressed in various tissues and are implicated in the etiology of many diseases (Kormish et al., 2010).

1.7.4. Sox5

Sox5 is a member of the SOXD group, which includes three genes, *sox5*, *sox6*, and *sox13* (Lefebvre, 2010). In adult testis, mouse Sox5 is expressed via a short transcript (2 kb) (Denny et al., 1992) whereas in other tissues and embryos it is expressed via a longer transcript (6 kb; Hiraoka et al., 1998). The short transcript encodes a 48-kDa protein isoform which lacks the N-terminal half of the larger protein encoded by the longer 6-kb transcript. The shorter Sox5 isoform was the first to be discovered and was named SOX5 (called Sox5S in this thesis; Harley and Lefebvre 2010). The longer Sox5 isoform was originally named L-SOX5 (Lefebvre et al., 1998), but most authors refer to this isoform as SOX5 (called Sox5L in this thesis). The longer Sox5 isoform is highly expressed in chondrocytes and striated muscles, which might play a role in human cartilage (Ikeda et al., 2002) and muscle development (Kou and Ikegawa, 2004). The phenotype of l-sox5 knock-out mice showed that L-SOX5 is necessary for i) the formation of cartilage, ii) the formation of the notochord sheath of extracellular matrix (Smits et al., 2001), iii) the survival of notochord cells (Smits and Lefebvre, 2003), and iv) the development of the nucleus pulposus of intervertebral discs (Dy et al., 2008). Sox5S, a SRY-related HMG TF was identified in the transcriptional regulation of sperm-associated antigen 6 (SPAG6) (Kiselak et al., 2010). The up-regulation of Sox11b mRNA after spinal cord injury is mainly taking place in ependymal cells lining the central canal and in newly differentiating neuronal precursors (Guo et al., 2010).

1.7.5. Other transcription factors involved in ciliary gene expression

TF hepatocyte nuclear factor 1β (HNF1β) has been demonstrated to play a crucial part in the homoeostasis of postnatal liver, pancreas and kidney (Igarashi et al., 2005). HNF1β-knock-out mice showed severe polycystic kidneys (Gresh et al., 2004). Efficient Chromatin immunoprecipitation approaches have identified several ciliary genes, including *Pkhd1* (polycystic kidney and hepatic disease 1), *Pkd2* (polycystic kidney disease 2), *Ift88* (intraflagellar transport 88) and collectrin, as targets of HNF1β (Gresh et al., 2004), suggesting that HNF1β plays an important role in ciliogenesis in the kidneys (Hiesberger et al., 2005; Thomas et al., 2010).

FKH-2(forkhead 2), another member of the forkhead family of TF, has been demonstrated to be essential for the specification of the particular type of sensory cilium (Mukhopadhyay et al., 2007). IFT is regulated by FKH-2 in these neurons through controlling the level of expression of a kinesin associated protein (kap1). This protein is a kinesin-II motor subunit, specifically in AWB (Amphid wing “B” cells) neurons. Thus, whereas DAF-19 is essential for controlling the formation of all types of cilia, FKH-2 is required only for specifying a particular ciliary subtype.

1.8. Reporter systems

Reporter genes are widely used as a rapid and convenient means of measuring molecular genetic events such as regulation of promoters by TF. The monitoring of transcriptional regulation via coupling with reporter gene expression has been used extensively to investigate various biological processes (Wood, 1995). A reporter gene is a promoter-attached gene introduced into a biological system the expression of which generates a readily measurable or observable phenotype. This provides a convenient parameter that is correlated with the molecular events associated with genetic expression (Wood, 1995). Since this thesis is mainly focused on promoter studies, several reporter genes were tested for their efficiency and sensitivity especially when coupled with a gene-specific promoter. The four different reporter genes used in this thesis are: the secreted alkaline phosphatase

(SEAP) gene, the Renilla luciferase gene, the Firefly luciferase (FFL) gene, and the Gaussia luciferase (GSL) gene.

SEAP is one of the most commonly used reporter genes for monitoring in vivo processes. Normally, APs are membrane-bound enzymes, thus not secreted. By introducing a termination codon at a position preceding the sequence encoding the membrane anchoring domain, the native 513-amino acid cell-surface form was converted into a truncated (489-amino acid residues), fully active secretory protein (Berger et al., 1988). This recombinant reporter gene can be constitutively expressed and efficiently released from transfected cells (Tannous and Teng, 2011).

Renilla luciferase is a 36 kDa monomeric protein that does not require post-translational modification for activity (Matthews et al., 1977). It catalyzes the chemical reaction that is responsible for the green bioluminescence of *Renilla reniformis,* a soft coral also known as sea pansy and a member of the class Anthozoa (Cormier et al., 1975). The enzyme catalyzes the oxidation of coelenterazine to yield coelenteramide and blue light of 480 nm (Hori et al., 1973). Lorenz and coworkers (1991) cloned the cDNA for the Renilla luciferase enzyme, determined that the protein contains 311 amino acid residues and also expressed the cloned gene in *E.coli*. Subsequently, the same group demonstrated the expression of the cloned gene in mammalian cells transfected either transiently or stably and suggested its potential use as a marker in studies of mammalian gene expression (Lorenz at al., 1996). As a reporter Renilla luciferase offers many of the same benefits as FFL, but no particular advantage, and its assay chemistry is limited. The primary limitation is the presence of a low level nonenzymatic luminescence, termed autoluminiscence, which reduces the assay sensitivity (Ruecker et al., 2008). Thus, Renilla luciferase is not generally preferred over FFL but it has recently become popular as a companion reporter for experiments in which two different reporters are needed (Alcaraz-Pérez et al., 2008).

Firefly (*Photinus pyralis*) luciferase is by far the most commonly used bioluminescent reporter due to both sensitivity and convenience of the enzyme assay and to the tight coupling of protein synthesis with enzyme activity (de Wet et al., 1985, 1987; Ow et al., 1986). FFL is a monomeric enzyme of 61 kDa which catalyzes a two-step oxidation reaction to yield light, usually in the green to yellow region, typically 550-570 nm. The first step is

activation of luciferyl carboxylate by ATP to yield a reactive mixed anhydride. In the second step, this activated intermediate reacts with oxygen to create a transient dioxetane that breaks down to the oxidized products, oxyluciferin and CO_2 (Gould and Subramani, 1988).

GSL is an extracellular luciferase isolated from the marine copepod *Gaussia princeps* (Verhaegent and Christopoulos, 2002). It is a small single chain polypeptide of only 185 amino acid residues that has attracted attention as a luciferase with superior properties when compared to the Renilla luciferase (Ruecker et al., 2008). GSL possesses a secretory signal consisting of 16 amino acid residues and therefore, upon expression in mammalian cells, is naturally secreted in an active form (Badr et al., 2007). GSL catalyzes the oxidation of the substrate coelenterazine in a reaction that is accompanied by emission of light. The GSL gene has been heterologously expressed in *Escherichia coli*, *Mycobacterium smegmatis* (Wiles et al., 2005), and in a codon-optimized form in mammalian cells (Tannous et al., 2005). All these studies describe *Gaussia* luciferase as a reliable reporter, with a higher signal intensity compared to other luciferases, and with enhanced stability in respect to challenging experimental conditions.

1.8.1. Dual reporter system

The term “dual reporter” refers to the simultaneous expression of two individual reporter enzymes within a single system. The limitation of using a single reporter assay is that under some experimental conditions changes in luminescence can be caused by factors other than the transcriptional control that is being studied, e.g., experimental discrepancies due to well-to-well variability in transfection efficiency. In order to overcome this limitation, a dual reporter system (Sherf et al, 1996) was used in all the deletion experiments in the present thesis. The dual reporter assay consisted of: 1) the FFL gene placed downstream from the response element under study, and 2) a second reporter, the GSL gene controlled by the full length wdr16 promoter, served as the internal standard for the transfection efficiency that varied from one experiment to another.

1.9. Aim of the thesis

The aim of the thesis is to characterize the wdr16 promoter. The genomic sequence interspersed between the first exon of the rat wdr16 and stx8 genes was found to act, in the direction of wdr16 transcription, as a promoter specifically activated in kinocilia-bearing cells at the time of kinocilia formation. This finding is the basis of this thesis on the characterization of the wdr16 promoter by identifying both the transcription factor binding site and the transcription factor, which regulates the promoter.

2. Results

2.1 Characterization of the promoter of the gene for the kinocilia-specific protein Wdr16

The orthologues of the wdr16 gene are especially suited as a paradigm for the analysis of kinocilia-specific promoters. They exhibit a special form of shared synteny, in which the wdr16 gene lies in "head-to-head" orientation to the stx8 gene with less than 1 kb separating the two genes. Due to the proximity of the adjacent stx8 gene, all major promoter elements are expected to be contained within the full length sequence of 766 nucleotides employed as the wild type promoter in this work (Fig. 1).

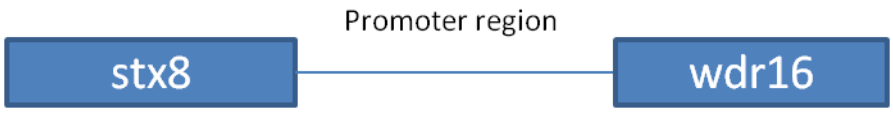


Fig. 1. Inverse genomic arrangement of the genes stx8 and wdr16 in relation to the region containing the promoters of these genes.

2.1.2. Deletion analysis of wdr16 promoter by using SEAP and FFL as reporters

Lentiviral vectors were designed which were bearing the genes for the reporters SEAP and FFL. The expression of the FFL gene under the full length promoter served as the reference for the experiments in which the FFL gene was under the control of partially deleted promoters. On this basis, four vectors were produced: 1) CTRL (both the SEAP gene and the FFL gene under the controls of the full length 766 bp long wdr16 promoters); 2) C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667); 3) C4 (the wdr16 promoter in front of the FFL gene had its first 400 bp deleted, i.e., sequence positions -766 to -367); and 4) C5 (the wdr16 promoter in front of the FFL gene was curtailed by its first 500 bp, i.e., sequence positions -766 to -267). EPC were infected with the CTRL viral vector at an MOI of 10 and analysed for their SEAP and FFL

synthesis. No SEAP activity was detected (data not shown). Therefore, the intended normalisation of the FFL activity between the individual viral transfection experiments could not be carried out and alternative reporters had to be taken into consideration.

2.1.3. Deletion analysis of wdr16 promoter by using the combination of Renilla and Firefly luciferase genes as reporter genes

As reported above no SEAP acitivity was detected in the culture medium, even when the EPC were transfected with the viral plasmid containing the full length wdr16 promoter. Therefore, SEAP was replaced by another reporter enzyme, Renilla luciferase. Thus, under the control of the full length wdr16 promoter Renilla luciferase served as the internal standard for the transfection effiency that varied from one experiment to another. On this basis, four vectors were produced: 1) CTRL (both the Renilla gene and the FFL gene under the controls of the full length 766 bp long wdr16 promoters) 2) C1 (the Renilla gene under the control of the full length wdr16 promoter, and the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667); 3) C4 (the Renilla gene under the control of the full length wdr16 promoter, and the wdr16 promoter in front of the FFL gene had its first 400 bp deleted, i.e., sequence positions -766 to -367), and 4) C5 (the Renilla gene under the control of the full length wdr16 promoter, and the wdr16 promoter in front of the FFL gene was curtailed by its first 500 bp, i.e., sequence positions -766 to -267). EPC were infected with the CTRL viral vector at an MOI of 10 and analysed for their Renilla luciferase and FFL synthesis. Analogous to the situation with SEAP no activity was detected in the case of Renilla luciferase as reporter. Therefore the normalisation of the FFL activity between the individual viral transfection experiments could not be carried out with Renilla luciferase as reporter. Consequently still another reporter gene had to be tested.

2.1.4. Deletion analysis of wdr16 promoter by using the combination of Gaussia luciferase (GSL) and FFL genes as reporter genes

Analogous to the situation with SEAP no Renilla luciferase acitivity was detected, as described in 2.1.3. Therefore, Renilla luciferase was replaced by GSL. GSL under the control of the full length wdr16 promoter served as the internal standard for the transfection effiency that varied fron one experiment to another. On this basis, four vectors were produced: 1) CTRL (both the GSL gene and the FFL gene under the controls of the full length 766 bp long wdr16 promoters); 2) C1 (the GSL gene under the control of the full length wdr16 promoter, and the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667); 3) C4 (the GSL gene under the control of the full length wdr16 promoter, and the wdr16 promoter in front of the FFL gene had its first 400 bp deleted, i.e., sequence positions -766 to -367); and 4) C5 (the GSL gene under the control of the full length wdr16 promoter, and the wdr16 promoter in front of the FFL gene was curtailed by its first 500 bp, i.e., sequence positions -766 to -267) It should be noted that in contrast to their meaning in 2.1.2 and 2.1.3, and starting with the present paragraph, C1, C4 and C5 designate constructs containing the GSL gene instead of genes for SEAP or Renilla luciferase. EPC were individually infected with one of the four viral vectors at an MOI of 10 and analysed for their GSL and FFL synthesis. Both GSL and FFL activity were detected. In comparison with the EPCs exposed to CTRL (Fig. 2), those infected by vector C1 (Fig. 3) contained only 10% of the FFL activity. EPCs treated with vector C4 (Fig. 4) eventually contained the same FFL activity as those from the C1 experiment, but expression of the FFL gene was delayed by more than 2 days in relation to C1-treated cultures. No FFL activity was detectable in EPCs treated with C5 (Fig. 5).

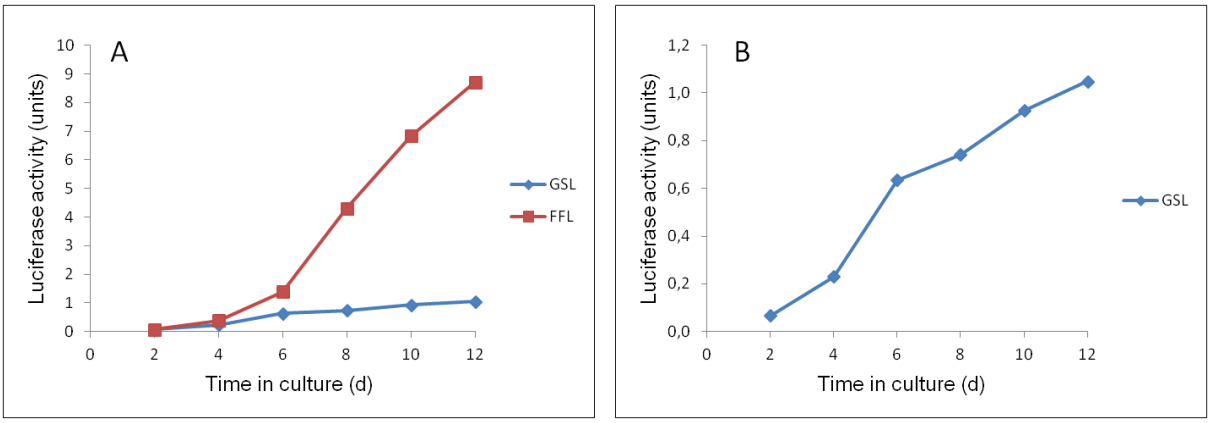


Fig. 2. Ependymal cultures (EPCs) were transfected with a lentiviral vector encoding GSL under the control of the full length wdr16 promoter as internal standard as well as the FFL gene under the control of the full length wdr16 promoter. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. The FFL activity served as positive control for the deletion analysis. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

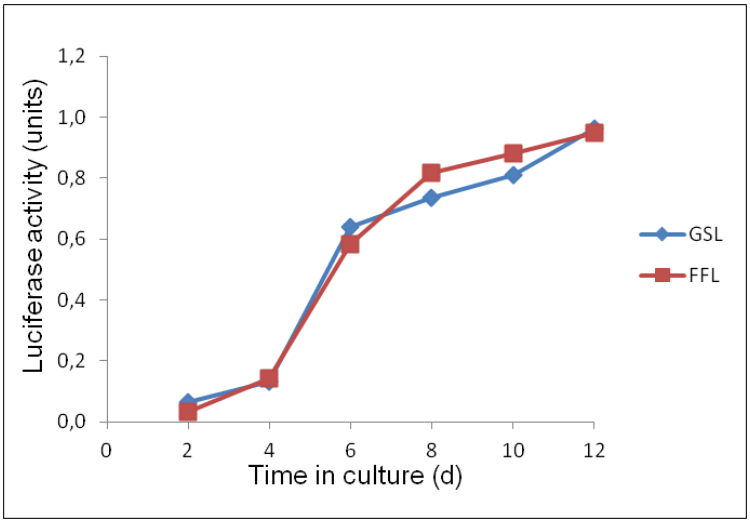


Fig. 3. EPCs were transfected with lentiviral vector C1 encoding GSL under the control of the full length wdr16 promoter as standard and the FFL gene under

the control of a wdr16 promoter in which the first 100 bp were deleted, i.e., sequence positions -766 to -667. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. The FFL activity showed a 90% decrease in comparison with that of the CTRL (Fig. 2).

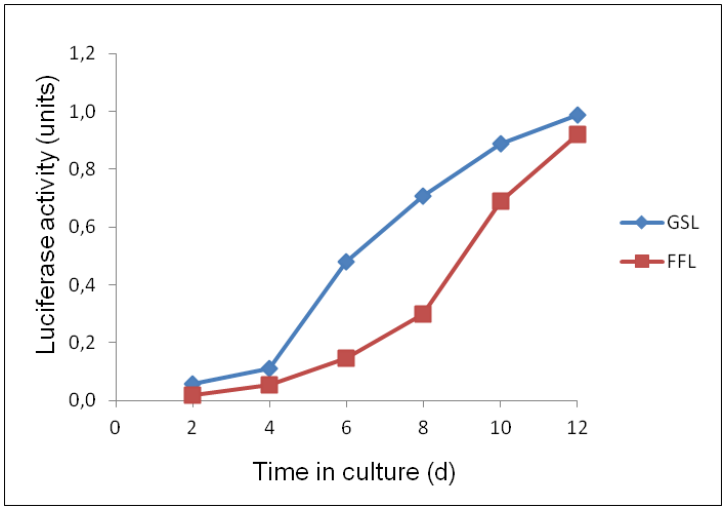


Fig. 4. EPCs were transfected with lentiviral vector C4 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 400 bp were curtailed, i.e., sequence positions -766 to -367. The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for firefly activity. Both GSL and FFL activity were detected. Although vector C4 gave rise to the same FFL activity as vector C1 (Fig. 3), appearance of FFL activity was delayed by more than 2 d.

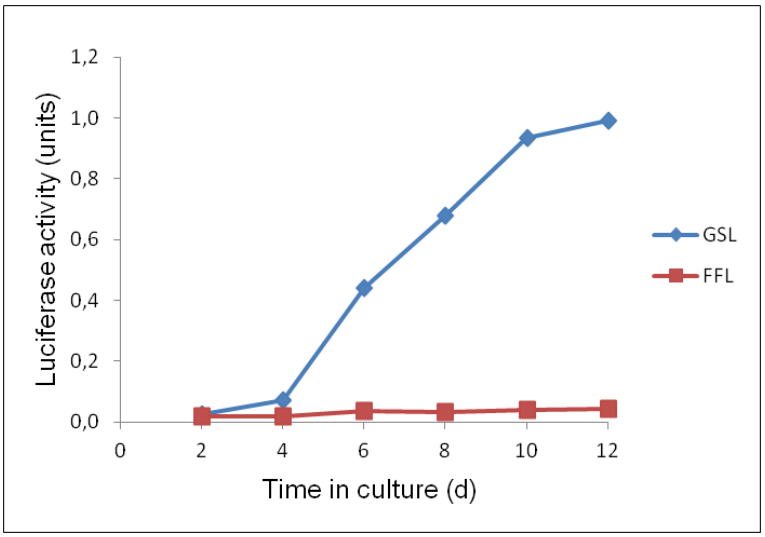


Fig. 5. EPCs were transfected with lentiviral vector C5 encoding GSL under the control of full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 500 bp were deleted, i.e., sequence positions -766 to -267. The medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. No FFL activity was detected but the internal standard GSL showed the same level of expression as in the experiment with the CTRL vector (Fig. 2).

2.1.5. Bioinformatics analysis for the full length promoter of wdr16

The 766 nucleotides of DNA sequence employed as the wdr16 promoter in the viral transduction experiments were analysed for conserved putative TFBSs in comparison to the orthologous mouse, macaque and human sequences via the computer programs FootPrinter software 3.0 and ConSite. The FootPrinter software detected 12 conserved motifs (CMs) of different length in the wdr16 promoter regions of the human, macaque, rat and mouse genes (Fig. 6a). Within the first eight CMs, the computer program ConSite identified binding sites for the transcription factors (TF) FREAC-4 (Foxd1), HFH-1 (Foxq1), HFH-2 (Foxd3), HFN3β (Foxa2), Sox5, SOX17, and Spz1 (Fig. 6b).

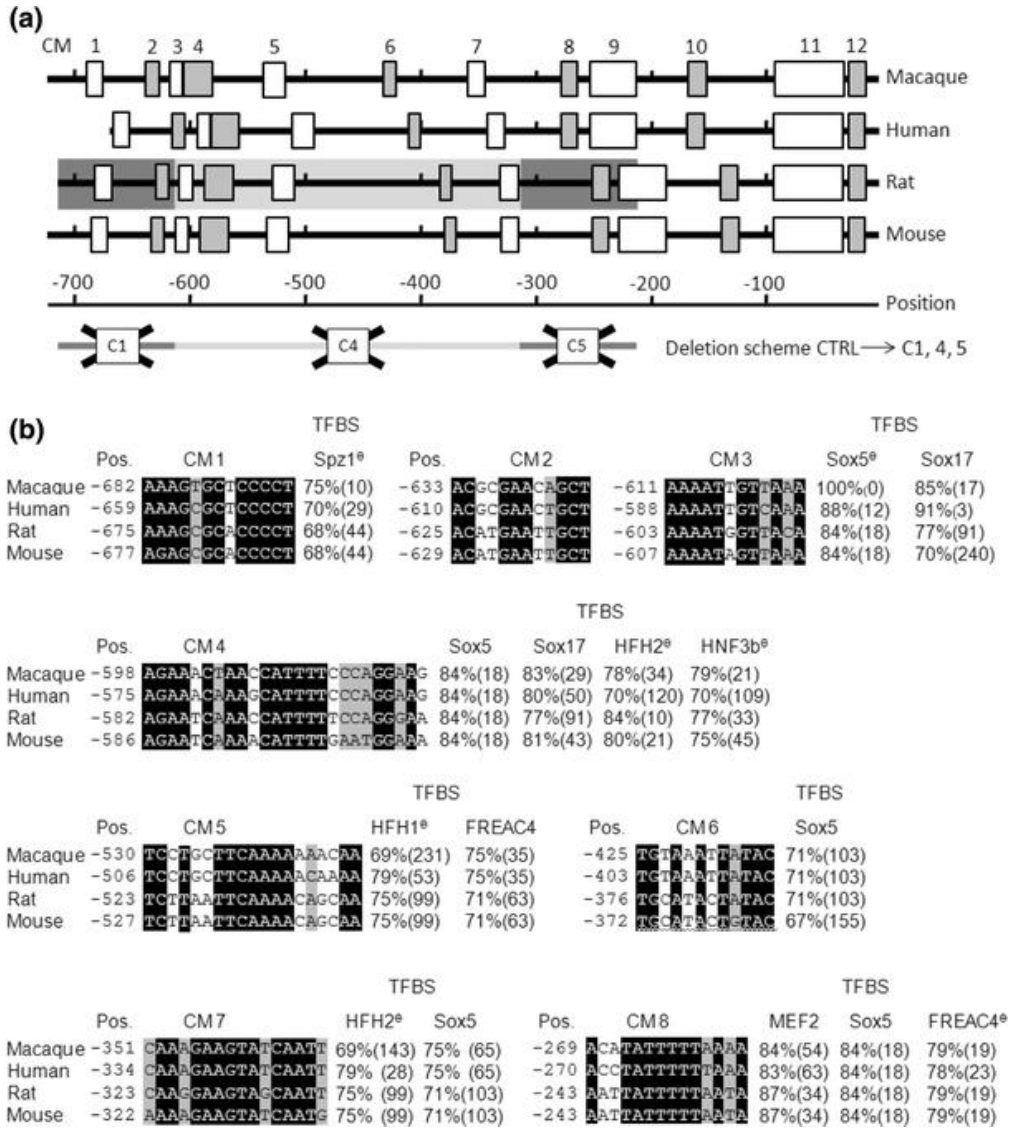


Fig. 6. The promoter regions of the macaque, human, rat and mouse orthologues of wdr16 exhibit conserved motifs (CMs) with putativeTF binding sites (TFBS). (**a)** CMs detected by the Footprinter 3.0 software (settings: motif size 12, maximum number of mutations 4, maximum mutations per branch 2, allow alignment guide, do not allow regulatory element losses) within the sequences are represented by alternating white and medium grey boxes numbered CM 1 through 12. The parts of the rat promoter region containing the sequence stretches that were consecutively deleted to transform CTRL into C1, C4, or C5, respectively, are highlighted by three consecutive boxes in dark grey, light grey and dark grey. The deletion scheme added below the position ruler connects these highlighting boxes to the designation of the respective deletion

product, either C1 in case of the absence of the sequence indicated by the first dark grey box, C4 in the absence of the sequences indicated by the first dark grey and the second light grey box, or C5 in the absence of the sequences indicated by all three boxes. (**b)** The detected CMs in the orthologous wdr16 promoters are aligned and labelled. The score for each putative TFBS identified by ConSite is indicated in terms of the percentage (%) of the maximal score of the respective position weight matrix (PWM) in the JASPAR database. The number in parentheses after this percentage score is the expected number of hits at this particular score within 1 kb of a random promoter sequence in the Eukaryotic Promoter Database. The superscript $^{\theta}$ at a TF abbreviation indicates that the binding site is on the complementary strand. To be included in this figure, a TFBS had to score at least an arbitrarily chosen 65% of the maximal PWM score in all four species. No putative TFBS was detected in the part of CM9 deleted in the transition from C4 to C5.

2.1.6. Characterization of the first 100 bp region by deletion analysis

Since the deletion of the first 100 bp region yielded a 90% decrease in the FFL activity (Fig. 3), further analysis of the first 100 bp was done by deletion analysis. For this, three more viral vectors were produced. 1) C1A (the wdr16 promoter in front of the FFL gene was stripped of its first 25 bp, i.e., sequence positions -766 to -742); 2) C1B (the wdr16 promoter in front of the FFL gene had its first 50 bp deleted, i.e., sequence positions -766 to -717); 3) C1C (the wdr16 promoter in front of the FFL gene was curtailed by its first 75 bp, i.e., sequence positions -766 to -692). Along with this the vectors CTRL (the FFL gene under the control of the full 766 bp long wdr16 promoter) and C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667) were used. In all cases the GSL gene put under the control of the full length wdr16 promoter served as the internal standard. EPCs were individually infected with each of the viral vectors at an MOI of 10 and analysed for their GSL and FFL synthesis. Both GSL and FFL activity were detected. In contrast to the EPCs exposed to CTRL (Fig. 6), those infected by C1A (Fig. 8), C1B (Fig. 9) and C1C (Fig. 10) did not show any decrease in the FFL activity, whereas in C1 (Fig. 11) the FFL activity was decreased by 90%.

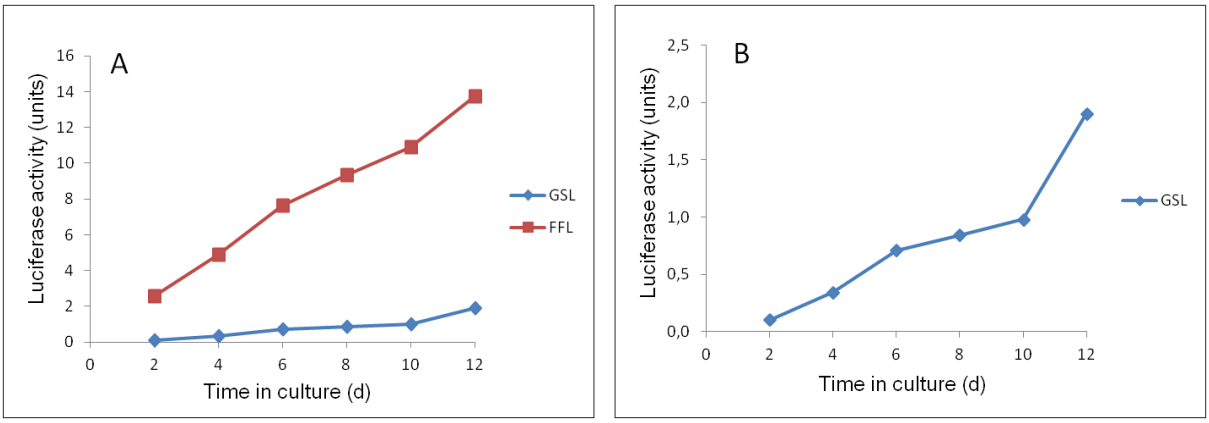


Fig. 7. EPCs were transfected with lentiviral vector CTRL encoding GSL under the control of the full length wdr16 promoter as internal standard as well as the FFL gene under the control of the full length wdr16 promoter. The medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. (A) The FFL activity shown in this figure served as a positive control for the deletion analysis of the 100 bp region to be shown in the subsequent figures. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

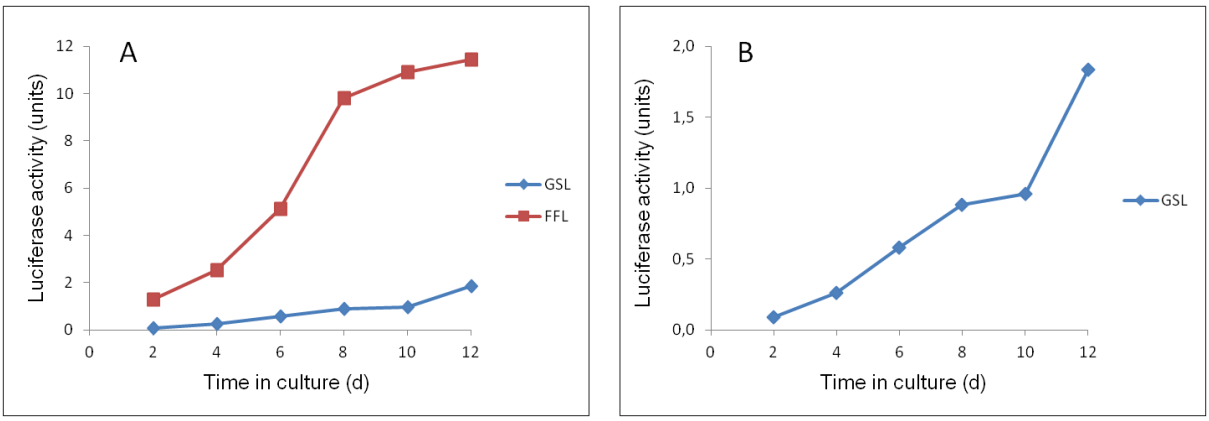


Fig. 8. EPCs were transfected with lentiviral vector C1A encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene

under the control of the wdr16 promoter in which the first 25 bp of the wdr16 full length promoter, i.e., sequence positions -766 to -742, were deleted. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. The FFL activity showed practically the same level as the CTRL (Fig. 7). (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

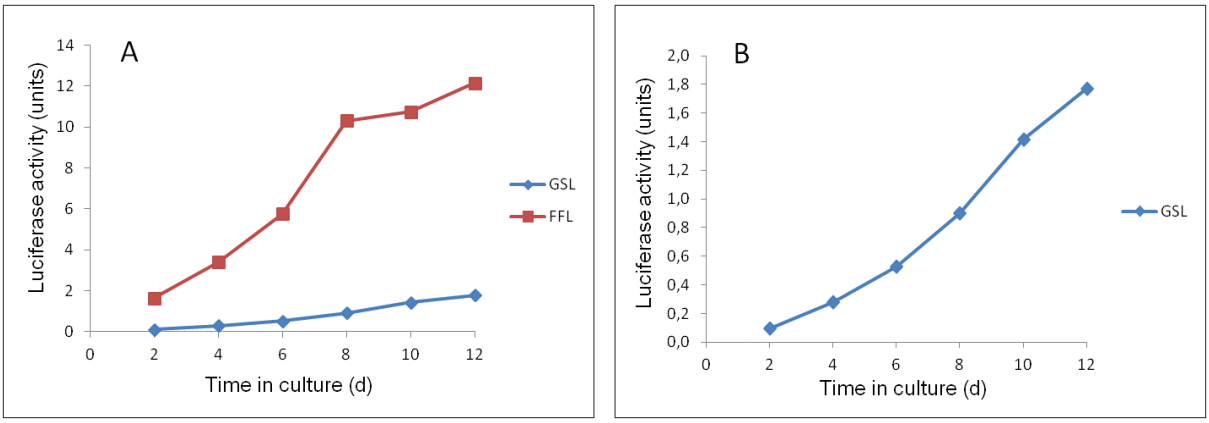


Fig. 9. EPCs were transfected with lentiviral vector C1B encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 50 bp were deleted from the full length promoter, i.e., sequence positions -766 to -717. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

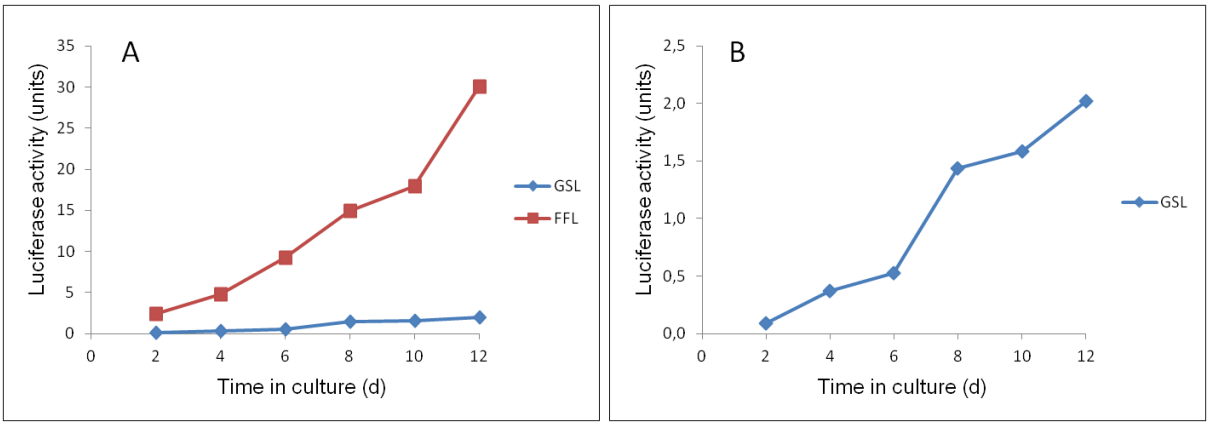


Fig. 10. EPCs were transfected with lentiviral vector C1C encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 75 bp of the full length promoter, i.e., sequence positions -766 to -692, were deleted. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activty and the cells were tested for FFL activity. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

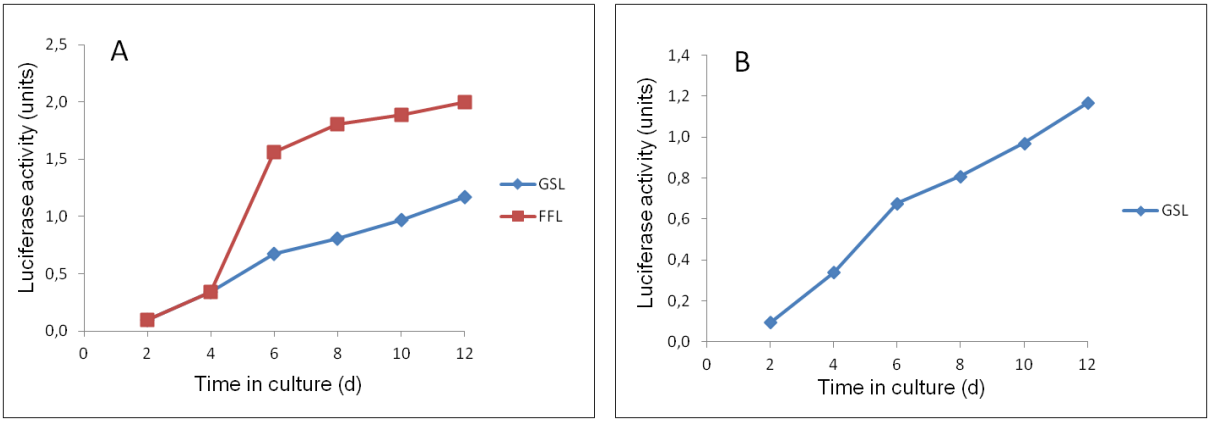


Fig. 11. EPCs were transfected with lentiviral vector C1 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of a promoter in which the first 100 bp of the full length wdr16 promoter, i.e., sequence positions -766 to -667, were deleted. The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and

DIV12 for the analysis of GSL and FFL synthesis. (A) The medium was tested for GSL activity and the cells were tested for FFL activity. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

2.1.7. Bioinformatics analysis of the core promoter region wdr16CPR

The core promoter region (last 25 bp of the first 100 bp region of the full length wdr16 promoter, i.e., sequence positions -691 to -667) were analysed for conserved putative TFBSs by the computer program ConSite, The computer program identified one binding site for Sox5. This is the only binding site identified in the 25 bp region.

2.1.8. Analysis of the promoter binding site in the core promoter region wdr16CPR

The deletion of the last 25 bp of the first 100 bp region, i.e., sequence positions -691 to -667, yielded a 90% decrease in FFL activity. Further analysis of the 25 bp was carried out to more precisely narrow down the promoter binding site by further deletion analysis. For this, four viral vectors were produced. 1) C1C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 80 bp, i.e., sequence positions -766 to -687); 2) C1C2 (the wdr16 promoter in front of the FFL gene had its first 85 bp deleted, i.e., sequence positions -766 to -682); 3) C1C3 (the wdr16 promoter in front of the FFL gene was curtailed by its first 90 bp, i.e., sequence positions -766 to -677); 4) C1C4 (the wdr16 promoter in front of the FFL gene was stripped of its first 95 bp, i.e., sequence positions -766 to -672). Along with these the vectors CTRL (the FFL gene under the control of the full 766 bp long wdr16 promoter) and C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667) were used. In all cases GSL which was put under the control of the full length wdr16 promoter served as the internal standard. EPCs were individually infected with each viral vector at an MOI of 10 and analysed for their GSL and FFL synthesis. Both GSL and FFL activity were detected. It should be stressed that, beginning with the experiments underlying Fig. 11, a new luminometer became available that was a 100 times more sensitive than the one used for the preceding experiments underlying Figs.

1-4 and Figs. 6-10. This explains the differences in the ordinate values obtained with the old and the new luminometer. In comparison with the EPCs exposed to CTRL (Fig. 11), those infected by C1C1 (Fig. 13), C1C2 (Fig. 14), or C1C3 (Fig. 15) did not show any decrease in the FFL activity, whereas the EPCs treated with C1C4 (Fig. 16) and C1 (Fig. 17) showed an about 60-70 % decrease in their FFL activity.

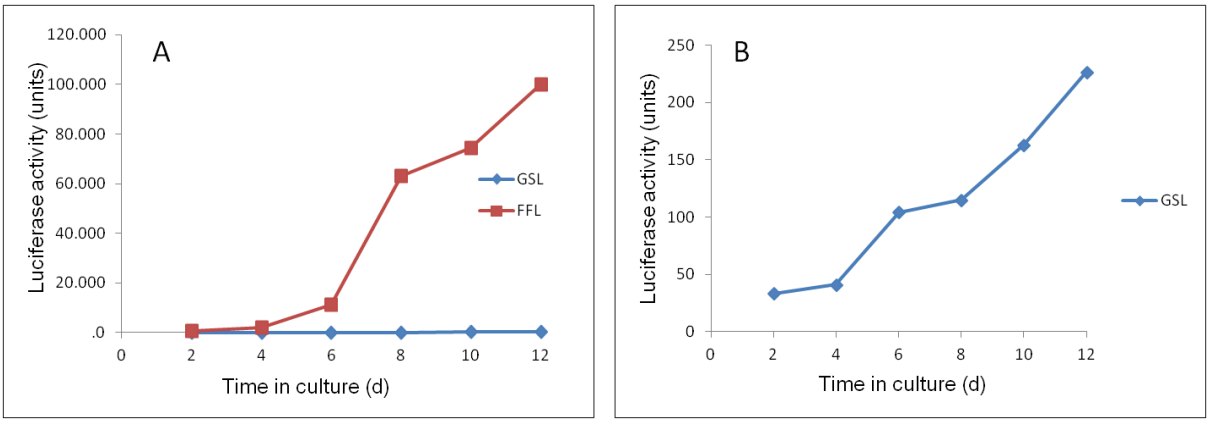


Fig. 12. EPCs were transfected with lentiviral vector CTRL encoding GSL under the control of the full length wdr16 promoter as internal standard as well as the FFL gene under the control of the full length wdr16 promoter. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and cells were tested for FFL activity. Both GSL and FFL activity were detected. This experiment served as the control for the 5 bp deletion analysis of the core promoter region. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

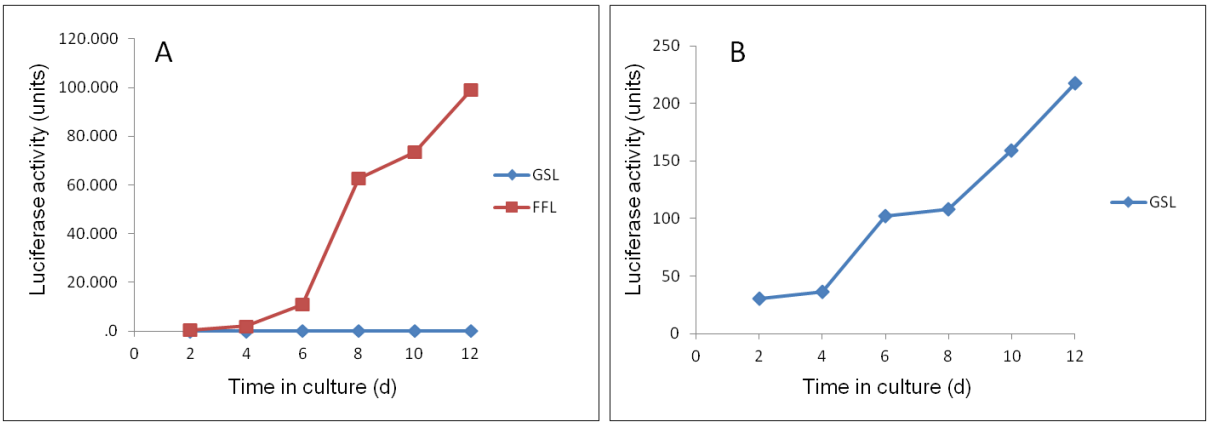


Fig. 13. EPCs were transfected with lentiviral vector C1C1 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 80 bp of the full length promoter were deleted, i.e., sequence positions -766 to -687. (A) The medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activty and the cells were tested for FFL activity. Both GSL and FFL activity were detected. The FFL activity shown is practically the same as that of the CTRL (Fig. 12). This demonstrates that the deletion of the first 5 bp of the core promoter region wdr16CPR did not affect the FFL activity. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

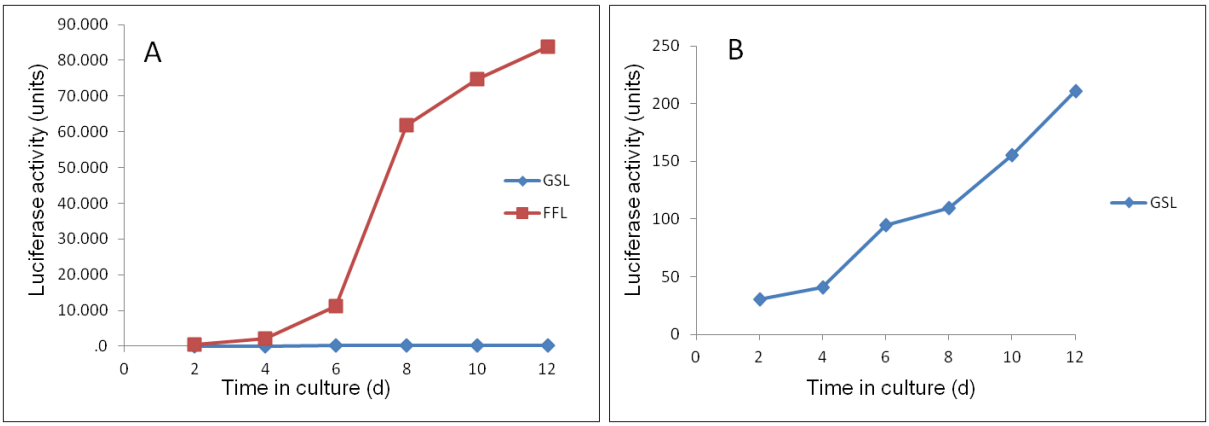


Fig. 14. EPCs were transfected with lentiviral vector C1C2 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL

gene under the control of the wdr16 promoter in which the first 85 bp of the full length promoter were deleted, i.e., sequence positions -766 to -682. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and cells were tested for FFL activity. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

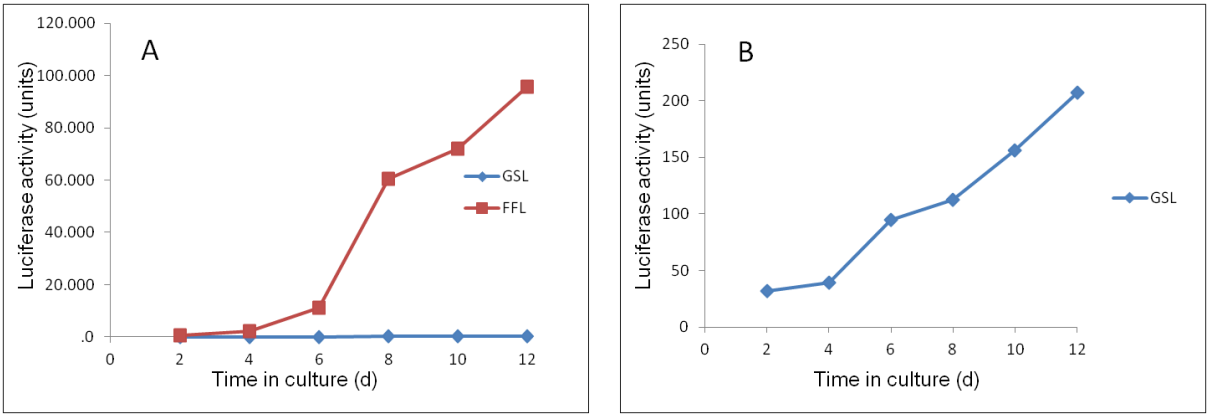


Fig. 15. EPCs were transfected with lentiviral vector C1C3 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 90 bp of the full length promoter were deleted, i.e., sequence positions -766 to -677. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments

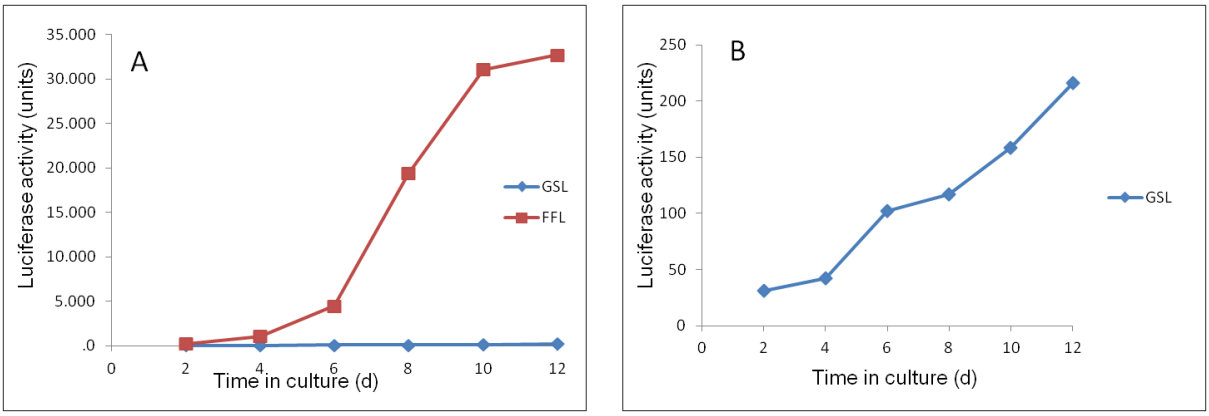


Fig. 16. EPCs were transfected with lentiviral vector C1C4 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 95 bp of the full length promoter were deleted, i.e., sequence positions -766 to -672. (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. In comparison to CTRL (Fig. 12) there was an approximately 65 % decrease in FFL activity. This shows that the deletion of the last 10 bp of the core promoter region wdr16CPR causes a considerable lose of FFL activity. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

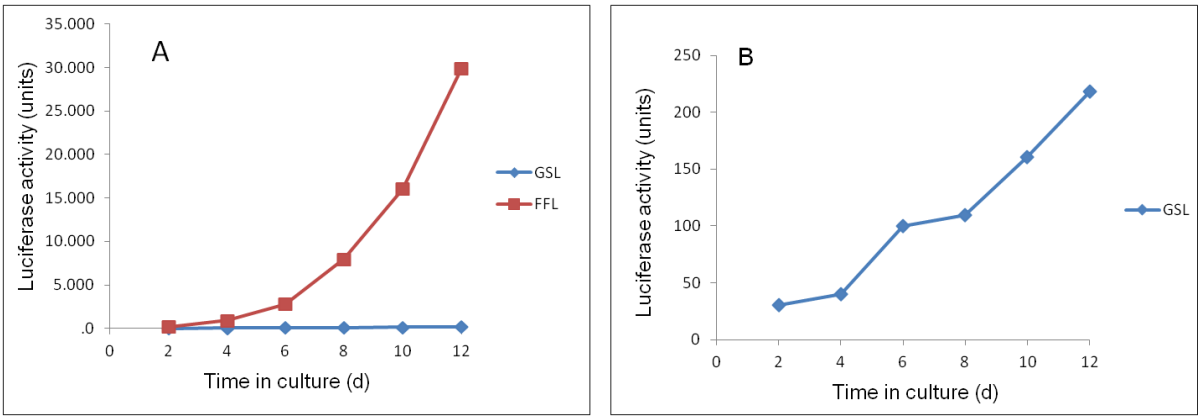


Fig. 17. EPCs were transfected with lentiviral vector C1 encoding GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene

under the control of the wdr16 promoter in which the first 100 bp of the full length promoter were deleted, i.e., sequence positions -766 to -667. (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. Both GSL and FFL activity were detected. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

The results obtained in the deletion analysis experiments (Figs. 1 to 4 and Figs. 6 to 16) are summarized in Table 1.

Table 1. Synopsis of the results obtained in the experiments with deleted wdr16 promoter

Vector construct	Length of deletion (bp)	Remaining length of promoter (bp)	Deleted sequence positions	Promoter positions present	% of maximal FFL activity	Remark	Fig. #
CTRL	0	766	0	-766 to -1	100		1
C1	100	666	-766 to -667	-666 to -1	10		2
C4	400	366	-766 to -367	-366 to -1	10	Delayed response	3
C5	500	266	-766 to - 267	-266 to -1	0		4
CTRL	0	766	0	-766 to -1	100		6
C1A	25	741	-766 to -742	-741 to -1	100		7
C1B	50	716	-766 to -717	-716 to -1	100		8
C1C	75	691	-766 to -692	-691 to -1	100		9
C1	100	666	-766 to -667	-666 to -1	10		10
CTRL	0	766	0	-766 to -1	100		11
C1C1	80	686	-766 to -687	-686 to -1	100		12
C1C2	85	681	-766 to -682	-681 to -1	100		13
C1C3	90	676	-766 to -677	-676 to -1	100		14
C1C4	95	671	-766 to -672	-671 to -1	35		15
C1	100	666	-766 to -667	-666 to -1	35		16

2.2. Infection of HEK293T cells with the full length wdr16 promoter

In order to demonstrate that wdr16 expression is specific only for ciliated cells, non ciliated cells such as HEK293T cells would not be expected to express the wdr16 gene. Thus, as

expected, when HEK293T cells were infected with the viral vector containing the reporter gene FFL under the control of the full length wdr16 promoter no FFL activity could be detected (data not shown).

2.2.1. Turn around of wdr16 full length promoter

To assess the proper orientation for wdr16 promoter function, CTRL (the FFL gene under the control of the full 766 bp long wdr16 promoter) was modified to INV, which contained the sequence (full length 766 bp) inserted in the opposite direction before the reading frame of the FFL gene and GSL under the control of the full length wdr16 promoter as internal standard. EPC infected with INV exhibited GSL as those treated with CTRL, but contained no measurable FFL activity (Fig. 18).

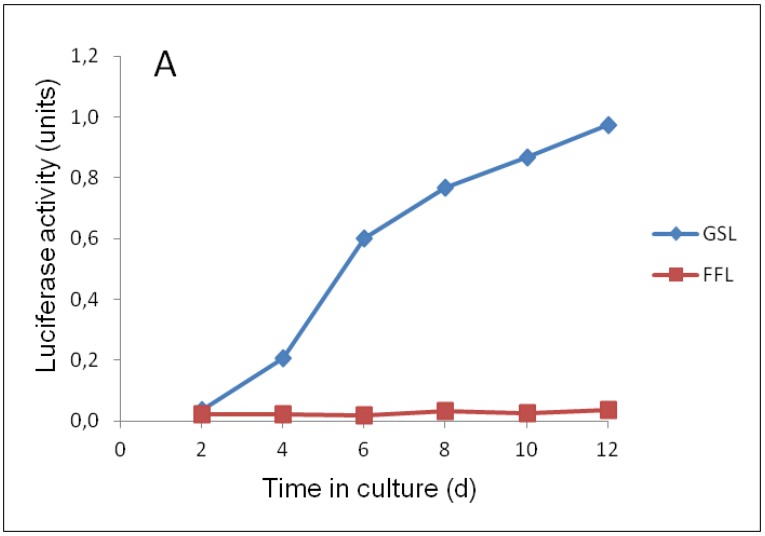


Fig. 18. EPCs were transfected with the viral vector INV which contained i) the 766 bp of the promoter controlling the FFL gene inserted in a direction opposite to the proper orientation, and ii) the properly oriented full length wdr16 promoter controlling the GSL gene. The medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activty and the cells were tested for FFL activity. There was no detectable FFL activity but GSL showed the same level of activity as that generated with CTRL.

2.3. Co-transfection of EPCs with wdr16 promoter DNA and sox5 DNA

The results obtained from the Bioinformatics analysis of the core promoter region wdr16CPR (last 25 bp of the first 100 bp, i.e., sequence positions -691 to -667) showed a binding site for Sox5. Therefore, viral vectors for sox5S (under the control of the EF1α promoter) and sox5L (under the control of the EF1α promoter) were produced. The viral vectors sox5S or sox5L were co-transfected on EPCs with the viral vector CTRL (the FFL gene under the control of the full 766 bp long wdr16 promoter). This was done inspite of the fact that EPCs have been shown to already express the gene for the TF Sox5. However it was not clear whether the level of this TF was sufficient for causing a maximum expression of the wdr16 gene. Therefore it was intended to raise in the EPCs the intracellular level of the TF by this co-transfection. Several control experiments were designed, such as 1) EPCs co-transfected with the viral vector sox5S (sox5S under the control of the EF1α promoter) and the viral vector C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667); 2) sox5L (sox5 L under the control of the EF1α promoter) with the viral vector C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667); 3) viral vector CTRL (the FFL gene under the control of the full 766 bp long wdr16 promoter) was co-transfected with a mock vector; 4) the viral vector C1 (the wdr16 promoter in front of the FFL gene was stripped of its first 100 bp, i.e., sequence positions -766 to -667) was co-transfected with a mock vector. The syntheses of GSL and FFL were analysed. The EPCs co-transfected with the viral vector CTRL and mock vector (Fig. 21) and the EPCs co-transfected with viral vector sox5S and viral vector CTRL (Fig. 19) produced practically the same level of FFL activity. There was a 30% increase in the level of FFL activity in the EPCs co-transfected with the viral vector sox5L instead of sox5S, and the viral vector CTRL (Fig. 20). In the EPCs co-transfected with viral vector C1 and mock vector the FFL and GSL activities were measured. Both activities were found (Fig. 22). The GSL and FFL activities of the EPCs co-transfected with the viral vectors C1 and sox5S or C1 and sox5L were measured. Both activities were found (Figs. 22 and 23). In comparison to the cultures cotransfected with the viral vectors CTRL and sox5L (Fig. 20), there was a 90 % decrease in FFL activity (Figs.22 and 23).

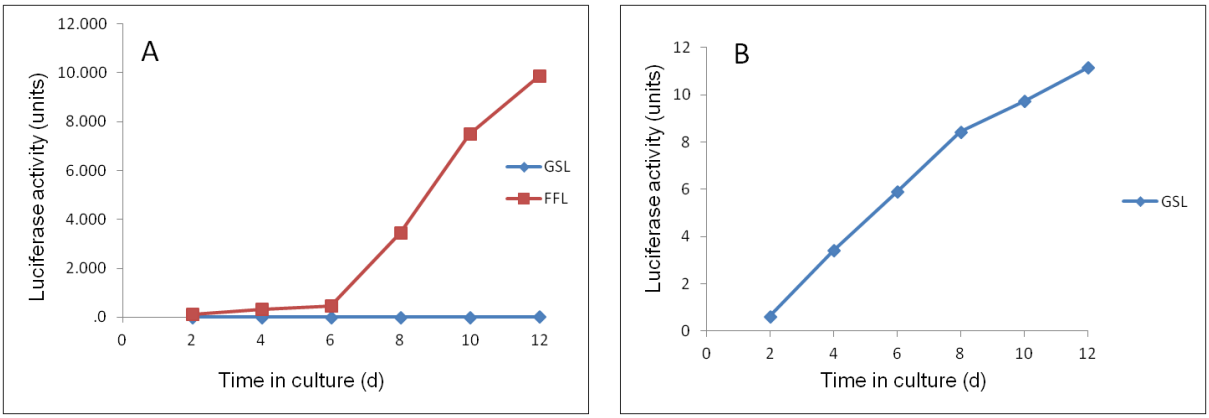


Fig. 19. EPCs were co-transfected with the lentiviral vectors CTRL (GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 full length promoter) and sox5S (under the control of the EF1α promoter). (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both activities were found (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

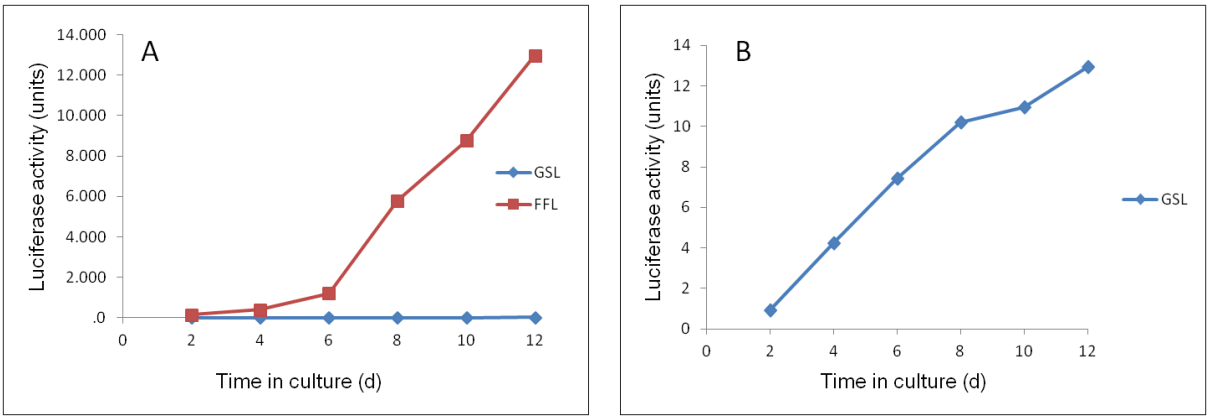


Fig. 20. EPCs were co-transfected with the lentiviral vectors CTRL (GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 full length promoter) and sox5L (under the control of the EF1α promoter). (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The

medium was tested for GSL activity and the cells were tested for FFL activity. Both activities were found. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

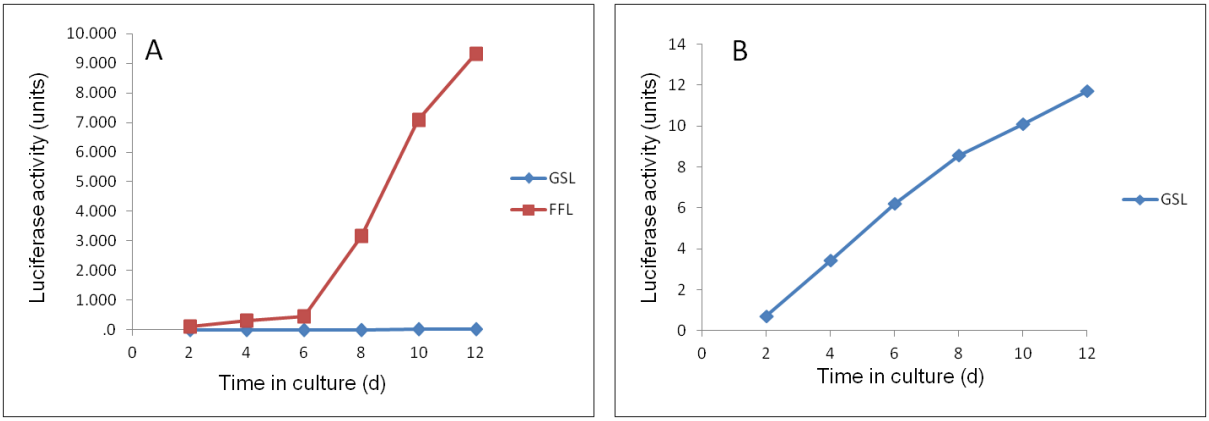


Fig. 21. EPCs were co-transfected with the lentiviral vectors CTRL (GSL under the control of full the length wdr16 promoter as internal standard and the FFL gene under the control of the full length wdr16 promoter) and the mock vector, which served as a negative control for analysing the expression level of wdr16 when co-transfected with sox5S or sox5L. (A) The medium and the cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments

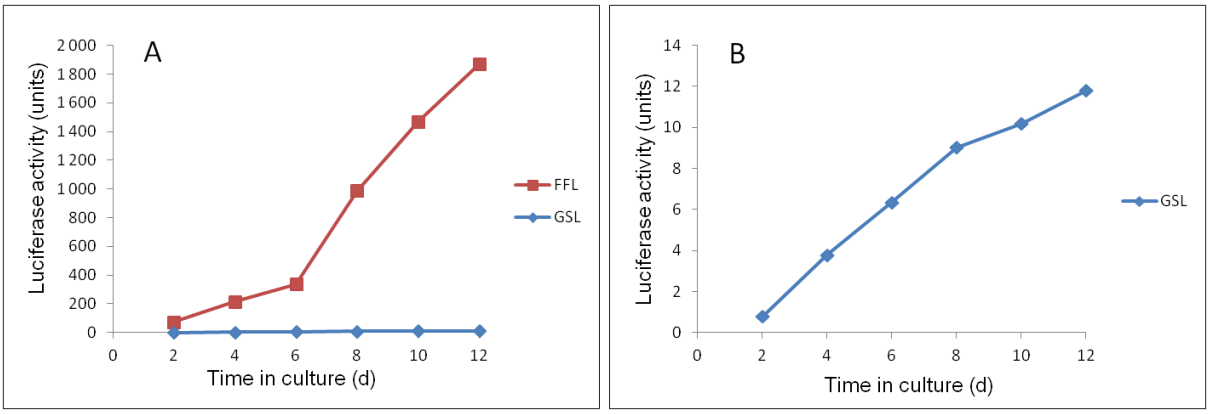


Fig. 22. EPCs were co-transfected with the lentiviral vectors C1 (GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 100 bp were deleted, i.e., sequence positions -766 to -667) and the mock vector. (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. In comparison to the CTRL (Fig. 21) there was an 80 % decrease in the FFL activity which shows that the first 100 bp region in the promoter plays a vital role in driving the expression of wdr16. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

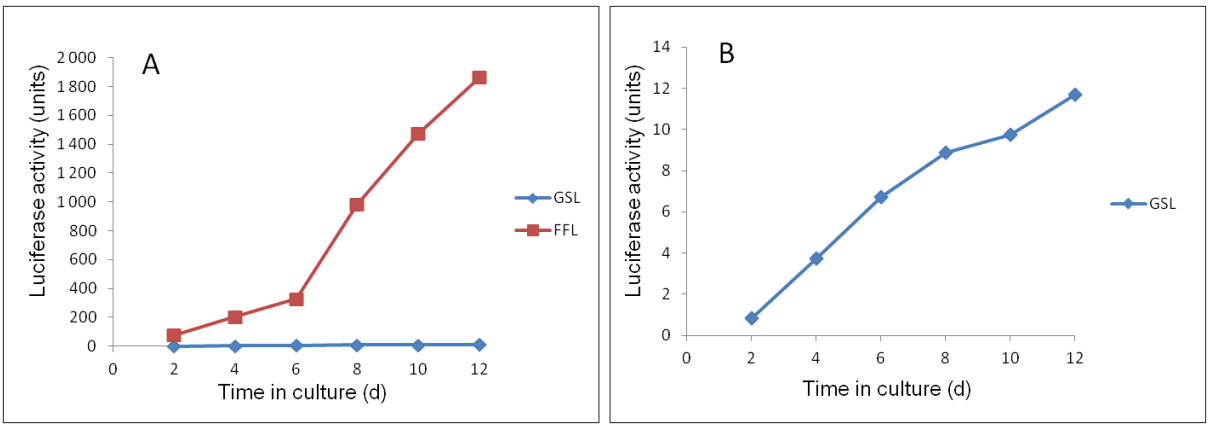


Fig. 23. EPCs were co-transfected with the lentiviral vectors C1 (GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene

under the control of the wdr16 promoter in which the first 100 bp were deleted, i.e., sequence positions -766 to -667) and sox5S (sox5S gene under the control of the EF1α promoter). (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. In comparison to the CTRL (Fig. 21) there was an 80 % decrease in the FFL activity which shows that the first 100 bp region in the promoter plays a vital role in driving the expression of wdr16. (B) Shows the GSL activity at higher resolution. The GSL activity was used to assess the constancy of transfection efficiency among individual viral transfection experiments.

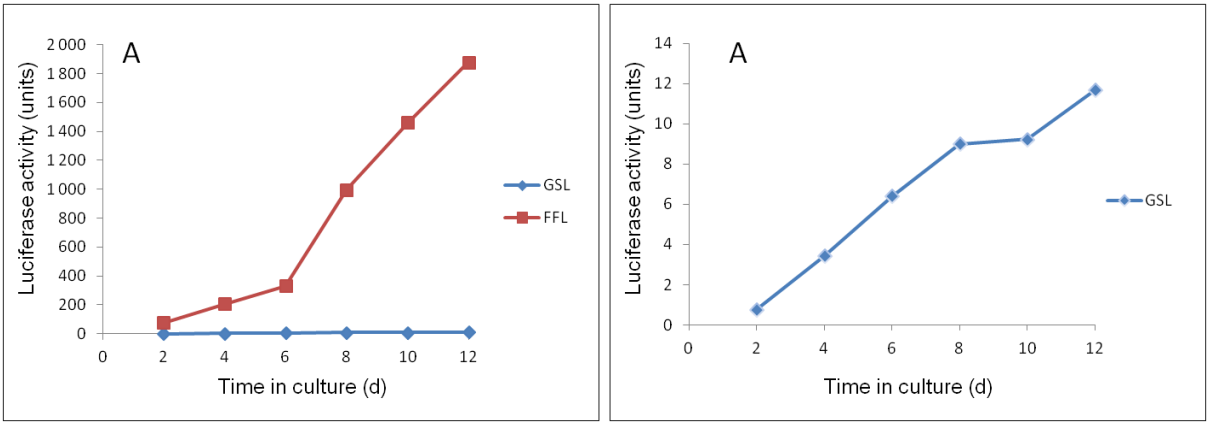


Fig. 24. EPCs were co-transfected with the lentiviral vectors C1 (GSL under the control of the full length wdr16 promoter as internal standard and the FFL gene under the control of the wdr16 promoter in which the first 100 bp were deleted, i.e., sequence positions -766 to -667) and the sox5L viral vector (sox5L under the control of the EF1α promoter). (A) Medium and cells were collected on DIV2, DIV4, DIV6, DIV8, DIV10 and DIV12 for the analysis of GSL and FFL synthesis. The medium was tested for GSL activity and the cells were tested for FFL activity. Both GSL and FFL activity were detected. In comparison to the cultures cotransfected with the viral vectors CTRL and sox5L, there was a 90 % decrease in the FFL activity. This shows that the first 100 bp region in the promoter has the binding site for Sox5 and it plays a vital role in driving the expression of wdr16. (B) Shows the GSL activity at higher resolution. The GSL activity was used

to assess the constancy of transfection efficiency among individual viral transfection experiments.

2.3.1 Analysis of the expression level of wdr16 in EPCs co-transfected with the wdr16 promoter and sox5 DNA

EPCs were co-transfected with the wdr16 promoter and the sox5 gene. Thereafter, Western blot analysis was done to assess the level of wdr16 expression; the densities of the bands were then compared with the results obtained from the luciferase assay (Figs. 18, 19, and 20). EPCs were co-transfected with the viral vectors sox5S or sox5L with the viral vector CTRL (the FFL gene under the control of the full 766 bp long wdr16 promoter). As controls, EPCs were co-transfected with viral vector CTRL and mock viral vector. The sox5S or sox5L genes were also co-transfected with the mock vector, and as a negative control HEK293T cell lysate was used. The EPC cells which were individually infected with each viral vector were collected and the homogenates from each culture were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes, which were then probed by an antibody against Wdr16. The intensities of the bands were quantified using AIDA Image analyser software. There was an about 30% increase in the amount of Wdr16 protein in the culture co-transfected with viral vectors CTRL and sox5L (Table 2; Fig. 25, lane 6) in comparison with EPCs co-transfected with CTRL and sox5S and the positive controls (Fig. 25, lanes 2-5).

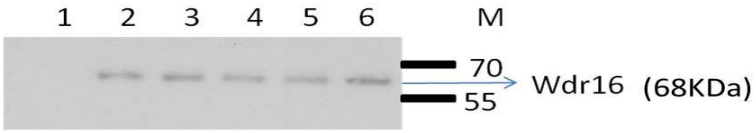


Fig. 25. EPCs were co-transfected with viral vectors CTRL and sox5S or sox5L. The cells were collected on DIV12. Homogenates from each culture were subjected to SDS PAGE (30 µg of protein per lane) and electroblotted onto nitrocellulose membranes, which were then probed by an antibody against Wdr16. Lane 1, HEK293T cell homogenate used as negative control; lane 2 represents the EPCs treated with CTRL and mock viral vector; lane 3 represents the EPCs treated with

sox5S and mock viral vector, lane 4 represents the EPCs treated with sox5L and mock viral vector; lane 5 represents the EPCs treated with CTRL and sox5S viral vector; and lane 6 represents the EPCs exposed to CTRL and sox5L viral vector. M, molecular mass standards.

Table 2. The intensity of the different bands of Wdr16 (Fig. 25) from the blot obtained from the EPCs co-transfected with the viral vectors CTRL and sox5 was quantified using AIDA Image analyzing software. Explicitly written the heading of the last column would be: 100 x (Intensity minus-background) / intensity (%).

Band number	Name of the sample	Area of the selected band (pixel)	Intensity (arbitrary unit)	Intensity minus background	Intensity minus background (%)
2	CTRL+Mock vector	14,330	3,710	950	25.6
3	sox5S + mock vector	14,330	3,780	1020	27.0
4	sox5L + mock vector	14,330	3,510	750	21.4
5	CTRL + sox5S	14,330	3,580	820	22.9
6	CTRL + sox5L	14,330	4,070	1310	32.2
	Background	14,330	2,760	0	0

2.4. Overexpression of sox5S and sox5L in HEK293T cells

For performing an electrophoretic mobility shift assay (EMSA) the sox5S and sox5L genes in the corresponding mammalian expression vectors were overexpressed in HEK293Tcells. The homogenates of the transfected cultures were subjected to SDS PAGE. Subsequently the proteins were electroblotted onto a nitrocellulose membrane, which was then probed by an antiflag antibody. The blot shows the expected bands for sox5S and sox5L at 48 and 84 kDa, respectively (Fig. 26).

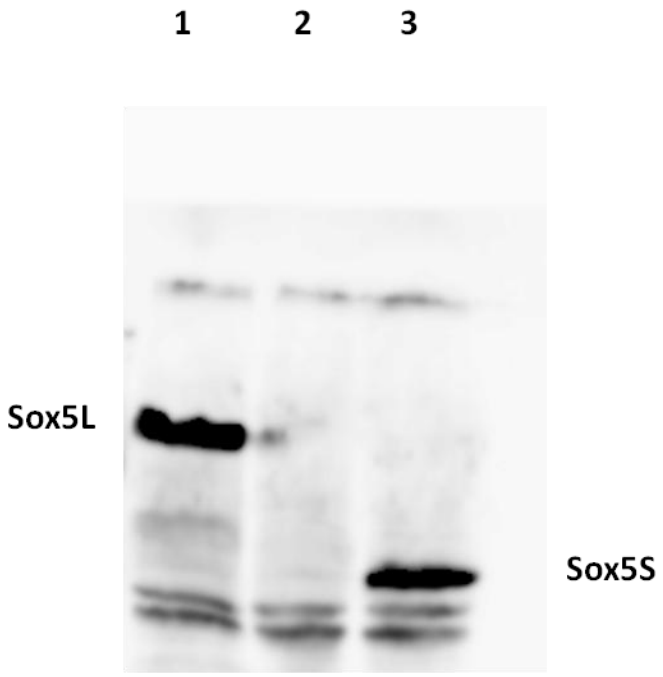


Fig. 26. Mammalian expression vectors for sox5S and sox5L genes were transfected into HEK293T cells. Homogenates prepared from each culture were subjected to SDS PAGE (30 µg of protein per lane). The proteins were electroblotted onto nitrocellulose membranes, which were then probed by an anti-flag antibody. Lane 1: represents overexpressed sox5L (84 kDa); lane 2: represents plain HEK293T cells which were used as negative control; lane 3: represents overexpressed sox5S (48 kDa).

2.5. EMSA for Sox5S and Sox5L

In order to detect the binding of Sox5 protein to the core promoter region wdr16CPR (last 25 bp of the first 100 bp in the full length wdr16 promoter, i.e., sequence positions -691 to -667), an EMSA was carried out. The nuclear extracts containing Sox5S and Sox5L were prepared from sox5 overexpressing HEK293T cells. The Wdr16CPR probe was synthesized by Sigma-Aldrich, Steinheim, and labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (4.2.8.2). For positive controls, sox5S and sox5L consensus site oligodeoxynucleotide probes were synthesized and labelled. To check if the observed shifted bands are specific for sox5, competition tests were run: to a protein extract that generated intense, shifted bands,

additionally to the labeled Wdr16CPR probe nonlabeled ("cold") oligonucleotide (Wdr16CPR) was added in excess. Plain HEK293T cells and the TF Specificity Protein 1 (SP1) were used for negative controls. Nuclear extracts were incubated with radioactively labeled oligonucleotide probes (Wdr16CPR, sox5S consensus site, sox5L consensus site). After the binding reaction had been carried out, the samples were separated by electrophoresis on a non-denaturing polyacrylamide gel and bands were detected by autoradiography. Wdr16CPR formed a distinct DNA-protein complex with the nuclear extracts from sox5S (Fig. 27, lane 6) and sox5L (Fig. 27, lane 7), like in the positive controls (Fig. 27, lanes 1 and 2). The bands for the competition tests were distinctly reduced in intensity (Fig. 27, lanes 8 and 9). This shows that the observed signals are sox5-specific. There was no shifted band in the negative controls.

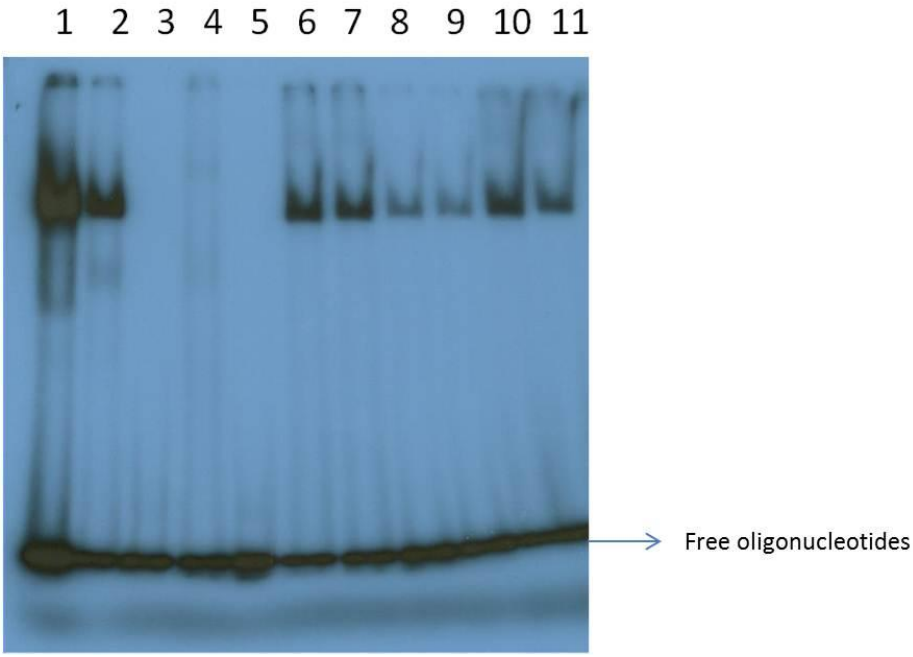


Fig. 27. An electrophoretic mobility shift assay was carried out for sox5S and sox5L DNAs with a Wdr16 probe labeled by [γ-^{32}P]ATP and T4 polynucleotide kinase. For positive controls a sox5S consensus site probe or a sox5L consensus site probe (4.2.8.2) was used. For negative controls the TF SP1 and nuclear extracts from HEK293T cells were used. For the competition test, additionally to the labeled Wdr16CPR probe, a nonlabeled ("cold") oligonucleotide (Wdr16CPR) was added in excess. Lane 1 shows Sox5S binding with the consensus site of

Sox5S. Lane 2 shows Sox5L binding with the Sox5L consensus site. Lanes 1 and 2 served as positive controls. Lane 3 represents the wdr16CPR probe with water containing no protein samples (negative control). Lane 4 represents the wdr16CPR probe with plain (untransformed) HEK293T cells (negative control). Lane 5 represents the wdr16CPR probe accompanied by SP1 (negative control). Lane 6 shows the complex of the wdr16CPR probe and Sox5S protein. Lane 7 shows the complex of the wdr16 WT probe and Sox5L protein. Lanes 8 and 9 (duplicates) represent the competition assay where unlabelled ("cold") wdr16CPR probe was added in excess. Lanes 10 and 11 are repetitions of lanes 6 and 7, respectively.

2.6. Yeast one hybrid

In order to identify and characterize the proteins that bind to the wdr16 promoter, a yeast one hybrid assay (Sieweke, 2000) was performed. The Yeast one hybrid assay helps to identify proteins that bind to a cis-acting DNA sequence as a target. Potential DNA-binding proteins, the prey, are expressed as fusion proteins with the GAL4 activation domain of pGADT7-Rec2. The target DNA sequence, or bait sequence, is cloned into pHIS2.1. Interaction between the DNA-binding protein and the target sequence stimulates transcription of HIS3, enabling the yeast host strain to grow on minimal media lacking histidine. The Matchmaker One-Hybrid library construction and screening kit (Clontech Laboratories) was used for performing the assay. The target DNA sequence, (bait sequence; full length 766 bp of the wdr16 promoter) was cloned into pHIS2.1 (one hybrid reporter vector that contains the HIS3 nutritional reporter gene which permits the cell to biosynthesize histidine and grow on a histidine minimal medium). Total EPC RNA was prepared and used for the generation of the cDNA required for making a library. The double-stranded cDNA synthesized from EPC RNA was then purified by using a CHROMA SPIN TE-400 column (Clontech laboratories) and was checked on an agarose gel where a smear of the DNA indicates the presence of double-stranded cDNA (Fig. 28). The concentration of purified cDNA obtained after purification was 4 µg/20 µl, a concentration sufficient for the subsequent step of transformation. Cotransformation was performed by

using 20 µl (4 µg) of the double stranded cDNA generated from EPC RNA, 6 µl of a solution of linear pGADT7-Rec2 (cloning vector used to have a protein of interest synthesized as a fusion protein containing also the GAL4 activation domain) and pHIS2.1 containing the wdr16 full length promoter. The transformation solution was spread in Triple Drop Out medium (TDO 3-AT) agar plates (SD/-His/-Leu/-Trp/50 mM 3-AT) and incubated for 4 d. Sixty four colonies were obtained from the cotransformation. In order to confirm the positive clones prior to sequencing, the colonies were restreaked on TDO 3-AT agar plates (SD/-His/-Leu/-Trp/50 mM 3-AT). After restreaking three times, there were only eight colonies left. The plasmid was isolated from all eight colonies and sequenced using T7 primer and Amplimer. The result of the sequencing did not indicate any gene responsible for binding between the wdr 16 promoter (bait) and protein(s) encoded by one or more member(s) of cDNA library (prey).

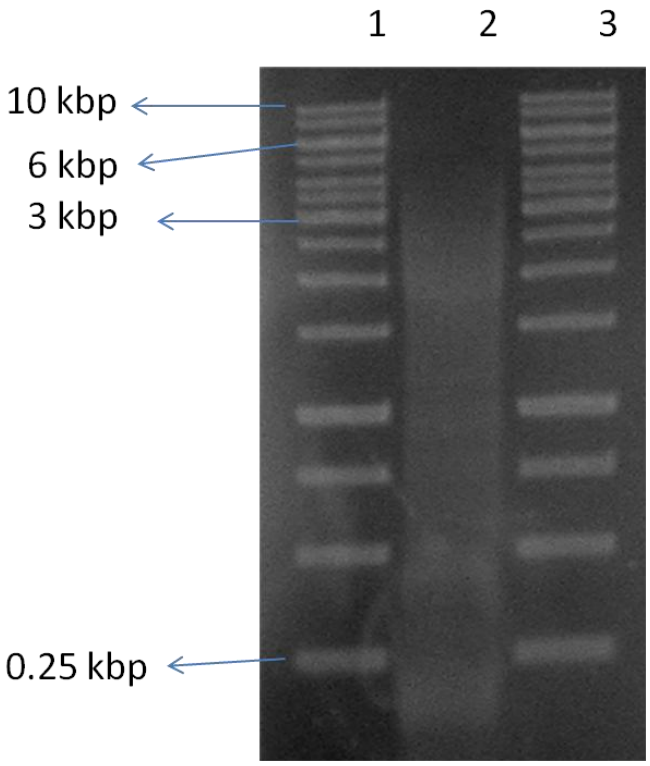


Fig. 28. Analytical agarose gel to check the double-stranded cDNA synthesized from total EPC RNA appears as a smear (lane 2). Lanes 1 and 3 show a DNA ladder consisting of the following bands from top to bottom: 10kbp, 8 kbp, 6 kbp, 5 kbp, 4 kbp, 3.5 kbp, 3 kbp, 2.5 kbp, 2 kbp, 1.5 kbp, 1 kbp, 0.75 kbp, 0.5 kbp, 0.25 kbp.

3. Discussion

3.1. Lentiviral vector-mediated gene transfer to ependymal primary cultures

Ependymal primary cultures are an established model for studying ependymal cell biochemistry and the biology of kinocilia-bearing cells (Verleysdonk, 2006b). However, the difficulty in causing them to express transgenes at high efficiency has been an important drawback of the system (Kowtharapu et al., 2009). plasmid-based transfection attempts remain at an efficiency below 1% and fail to elicit reporter gene expression, namely green fluorescent protein (GFP) synthesis, in any of the kinocilia-bearing cells of the cultures (Kowtharapu et al., 2009). This is partially explainable by the absence from the surface of the target cells of membrane lectins known to be required for the uptake of plasmid DNA in complex with cationic polymers or by impaired intracellular trafficking of the plasmid DNA/polymer complexes (Fajac et al., 2003). Ependymal cells are postmitotic (Spassky et al., 2005) and productive transfection requires the transfer of the foreign DNA to the nucleus. HIV-derived vectors infect non-dividing cells due to the karyophilic properties of the viral preintegration complex, which, after virus infection, is actively transported to the host cell nucleus. The work of Bhavani kowtharapu was the first to establish the transduction of genes into undifferentiated as well as differentiated EPC with lentiviral vectors. In his work (Kowtharapu et al., 2009) he showed that application of lentiviral vectors bearing the GFP gene as a marker resulted in a tremendous increase in general efficiency compared to transfection with a plasmid. The enhancement amounted to a factor of 75 with respect to undifferentiated EPCs and to a factor of 20 with respect to differentiated EPCs. This difference in transfection efficiency between differentiated and undifferentiated cells is similar to the one reported in a study about usage of a VSV-G-pseudotyped lentiviral vector on airway epithelial cells. Undifferentiated airway epithelia were efficiently infected, but the well-differentiated pseudostratified columnar epithelium of mature human bronchial xenografts was largely resistant to such infection (Goldman et al., 1997).

Cell-specific promoters are advantageous since they are less prone to suffer from promoter inactivation and less likely to activate the defense machinery of the host cell (Liu et al., 2004). Consequently, improved stability and longevity of gene expression can be expected.

To date, cell-specific gene expression has been described for several cell types, including erythroid (Moreau-Gaudry et al., 2001), endothelial (De Palma et al., 2003), central nervous system (Gascon et al., 2008; Greenberg et al., 2007; Kuroda et al., 2008; Lai and Brady 2002; Liu et al., 2008), retinal (Miyoshi et al., 1997; Semple-Rowland et al., 2007), liver (Oertel et al., 2003; VandenDriessche et al., 2002), and cancer (Morgan et al., 2006; Seo et al., 2009; Uch et al., 2003; Yu et al., 2001) cells. As shown in the present thesis, HIV pseudotyped with the vesicular stomatitis virus envelope glycoprotein (HIV/VSV-G) and encoding GFP under the control of the ubiquitously recognised promoter of elongation factor 1 alpha (EF1alpha) also does not cause transgene expression in the kinocilia-bearing cells of an EPC when applied at MOIs of up to 40 and destroys the culture when the MOI is increased further. In contrast, HIV/VSV-G encoding GFP under the control of a promoter specifically active in kinocilia-bearing cells leads to transgene expression in up to 79% of the kinociliated cells of an EPC when applied at an MOI of 20 (Kowtharapu et al., 2009). This result has permitted characterisation of the promoter of the kinocilia-specific gene wdr16 in the present work.

3.2. wdr16 promoter

The rat orthologue of wdr16 is a gene the product of which is only present in kinocilia-bearing, but absent from all other cells, even from those with a primary cilium (Hirschner et al., 2007). The orthologues of the wdr16 gene are especially suited as a paradigm for the analysis of kinocilia-specific promoters. They exhibit a special form of shared synteny, in which wdr16 lies in “head-to-head” orientation to the stx8 gene (Hirschner et al., 2007). The genomic sequence interspersed between the first exons of the rat wdr16 and stx8 genes was found to act, in the direction of wdr16 transcription, as a promoter specifically activated in kinocilia-bearing cells at the time of kinocilia formation (Kowtharapu et al., 2009). Infection of EPC by HIV/VSV-G/wdr16-p/GFP led to transgene expression in 79% of all kinociliated cells of the culture (Kowtharapu et al., 2009). This finding not only enables future researchers to express transgenes in ependymal cells with previously unachievable efficiency, but also permits the characterisation of the wdr16 promoter itself. Due to the proximity of the adjacent stx8 gene, all promoter elements are expected to be contained within the 766 bp. of sequence employed as the promoter in the present work. This idea is supported by the capacity of this sequence to drive GFP expression with a high efficacy

similar to that of the Foxj1 promoter. The transcription factor Foxj1, expressed in later stages of epithelial cell differentiation, is a known regulator of ciliogenesis (Blatt et al., 1999; Brody et al., 2000).

3.3. Gaussia luciferase is a sensitive reporter gene for monitoring the promoter activity

In order to normalize the FFL activity between individual viral transfection experiments dual reporter constructs were used for the deletion analysis experiments. To normalize, the SEAP gene was used as the second reporter gene. Since there was no detectable signal, the normalization could not be realized. Therefore, an alternative reporter had to be taken into consideration. Consequently, SEAP was replaced by Renilla luciferase. But inspite of this sensitive indicator system no signal could be detected. This might be due to the short in vitro half-life of the enzyme and the low detection limit associated with such a weak promoter (Heitzer and Zschoernig, 2007). Renilla luciferase was then replaced by GSL in which a sufficiently strong signal was obtained to normalize the FFL activity between individual viral transfection experiments. GSL is an extracellular luciferase isolated from the marine copepod *Gaussia princeps, a* crustacean (Verhaegent and Christopoulos, 2002). GSL was shown to contain two catalytic domains, which enables the enzyme to emit light in a highly efficient way and thus makes the Gaussia luciferase gene a higly sensitive reporter gene (Inouye and Sahara, 2008). GSL proved to be a quantifiable reporter superior to Renilla luciferase for the analysis of constitutive promoter sequences in *Chlamydomonas reinhardtii* (Ruecker et al., 2008). GSL possesses a natural secretory signal and upon expression is secreted into the cell medium. This secretion greatly facilitates the GSL assay. With respect to the challenging experimental conditions employed and in comparison to other luciferases, in the present work GSL presents itself as a higly useful reporter displaying high signal intensity and high storage stability.

3.4. Characterisation of wdr16 full-length promoter by bioinformatics analysis and initial deletion analysis experiments

Initial deletion analysis experiments were done by using four vectors, namely CTRL, C1, C4 and C5. In comparison with the EPCs exposed to CTRL, those transfected by C1 contained only 10% of the FFL activity (Fig. 3). EPC treated with C4 eventually contained the same FFL activity as those from the C1 experiment, but expression of the FFL gene was delayed by more than two days in relation to C1-treated cultures (Fig. 4). No FFL activity was detectable in EPC after infection with C5 (Fig. 5). Bioinformatics analysis of the wdr16 promoter region covered by the probe vectors CTRL, C1, C2, C4 and C5 revealed putative binding sites for eight different TF, including four of the forkhead group. While also the kinocilia-associated TF Foxj1 is a member of the forkhead family, a Foxj1 binding site was not identified in any CM. This may be seen as an indication that during kinociliogenesis Wdr16 protein does not appear after Foxj1, i.e., its presence is not dependent on the prior appearance of Foxj1. C5 did not exhibit any promoter activity in the FFL reporter gene assay, even though it contains the majority of CM 9 and CMs 10 to 12. The hypothetical binding of TF to these motifs is therefore insufficient to allow promoter activation. However, such TF could still interact with those present at BSs further upstream to modulate wdr16 transcription. In contrast to C5, C4 possessed measurable promoter activity in EPC, but with expression kinetics significantly different from C1 and CTRL. Within the 100 bp long additional sequence that is present in C4 but not in C5, the only CM identified by FootPrinter 3.0 is #8, which contains BSs for MEF2, Sox5 and FREAC-4. A score of 87% of the maximum for the MEF2 matrix as obtained at CM 8 will be observed 0.5 times in a random promoter sequence of the same length (14 bp), identifying the MEF2 PWM as rather unspecific. For Sox5 and FREAC-4, the expected numbers of hits based on a random promoter sequence of a length of 14 nucleotides are only about 0.3 each. The MADS-box TF MEF2 is required for myoblast differentiation and muscle morphogenesis (Cripps et al., 1999). It has been reported to control the expression of the *Drosophila* tubulin β3 gene, which is normally restricted to the mesoderm (Damm et al., 1998), as is MEF2 itself (Edmondson et al., 1994). Ependymal cells, however, are of neuroectodermal origin (Del Bigio, 1995; Spassky et al., 2005) and tubulin β3 does not normally occur in the kinociliary axoneme. In fact, ectopic expression of tubulin

β3 disrupts the axonemal architecture (Fackenthal et al., 1995). Therefore, it is likely that MEF2 does not physiologically bind to CM 8 to regulate the wdr16 promoter. FREAC-4 (Foxd1), on the other hand, is known to control Foxj1 expression (Lin and Peng, 2006), which, in turn, is essential for kinociliogenesis (Brody et al., 2000; Chen et al., 1998). FREAC-4 is therefore the candidate TF to regulate the wdr16 promoter at CM 8, and it may be responsible for the residual promoter activity in C4 as compared to C5. The HMG TF Sox5 is involved in the regulation of oligodendrocyte (Stolt et al., 2006) and chondrocyte (Smits et al., 2001) development, but also in spermatogenesis (Denny et al., 1992), S-SOX5 found to be involved in the motile/flagella through the control of expression of an axoneme central apparatus gene SPAG6 (Kiselak et al., 2010). Although its PWM extends over not more than seven nucleotides, the information content at each position is between 1.5 and 2 bits, putting its specificity on par with many PWMs encompassing more nucleotides. Possible Sox5 binding sites were found in five of eight CMs, making Sox5 a likely regulator of the wdr16 promoter.

C1 differed from CTRL by the lack of 100 bp of sequence, including CM 1 and CM 2, which caused the loss of 90% of FFL activity compared to CTRL. In CTRL and C1 the time courses of the expression of the FFL gene were very similar, suggesting that CMs 1 and 2 control promoter strength rather than specificity. The responsible TF remains elusive in case of CM 2, for which no TFBS could be identified. Analysis by software revealed that CM 1 was associated with Spz1 binding. The number of expected random hits within the 14 bp of CM 1 at 68% of the maximal score for the Spz1 matrix in the JASPAR database is 0.6. Spz1, a basic helix-loop-helix family TF originally isolated from a mouse testis library (Hsu et al., 2001), plays an important regulatory role during spermatogenesis (Hsu et al., 2004). Notably, the promoters of the genes encoding the axonemal proteins Pf6, Spag6 and Pf20 feature multiple putative Spz1 binding sites (Horowitz et al., 2005), implying Spz1 in the regulation of axoneme-related genes and making it a promising candidate for functional binding to CM 1. Next to the loss of the Spz1 site, the lack from C1 of the putative SOX17 BS identified by ConSite and CONREAL at position −660, but missed by FootPrinter and therefore not included in Fig. 6, may account for the dramatic drop in FFL activity between CTRL and C1. The Sry-related HMG TF SOX17 has been shown to regulate the Foxj1 promoter (Park et al., 2006), and it also induces the differentiation of cultured alveolar type II cells into cilia-

bearing, polarised epithelial cells (Park et al., 2006). SOX17 is therefore a very likely candidate as regulator of wdr16 transcription.

In the transformation of C1 into C4, 300 nucleotides of promoter sequence, containing motifs 3 through 7, were deleted, resulting in a delay of 2 days before reporter gene activation in EPCs. The CMs in the deleted region contain putative binding sites for the TF FREAC-5, HFH-1, HFH-2, HNF-3β, Sox5 and SOX17. HFH-1 (Foxq1) is present in epithelial cells (Hoggatt et al., 2000) but since there is no indication for the presence of HFH-1 in the brain (Choi et al., 2006; Hoggatt et al., 2000), the importance of HFH-1 for wdr16 expression needs to be further investigated experimentally. HFH-2 (Foxd3) promotes the development of neural crest cells from neural tube progenitors (Dottori et al., 2001). Therefore its involvement in ependymal differentiation appears possible. HNF-3β (Foxa2), together with Foxa1, has been shown to be required for the synthesis of the kinocilia-related TF Foxj1 (Wan et al., 2005). It is therefore a good candidate for wdr16 regulation. Notwithstanding the importance of CMs 3–7 for the proper kinetics of wdr16 expression, the most critical elements for wdr16 expression specifically in kinocilia-bearing cells appear to reside in the respective 100 bp long sequences deleted in the transitions from CTRL to C1 and from C4 to C5. The deletion of the 100 nucleotides long sequence in the transition from CTRL to C1 reduces the promoter strength by 90%, while removal of the 100 nucleotides long sequences in the transition from C4 to C5 area completely abrogates promoter activity. The corresponding DNA sequences, especially the CMs identified therein, offer themselves for further analysis concerning functional binding of TF, especially of Spz1, Sox5 and FREAC-4.

3.5. Characterization of the first 100 bp region of the full length promoter

Since the deletion of the first 100 bp region reduces the promoter strength by 90%, the 100 bp regions was further characterized by deletion analysis experiments. For this, three viral probe vectors were produced such as C1A (the wdr16 promoter in front of the FFL gene was stripped of its first 25 bp, i.e., sequence positions -766 to -742), C1B (the wdr16 promoter in front of the FFL gene had its first 50 bp deleted, i.e., sequence positions -766 to -717), and C1C (the wdr16 promoter in front of the FFL gene was curtailed by its first 75 bp, i.e., sequence positions -766 to -692) along with CTRL and C1. Compared to the FFL activity of

CTRL (Fig. 7) there was no decrease in the FFL activity if the construct C1A (Fig. 8), C1B (Fig. 9) or C1C (Fig. 10) was used, whereas in the presence of C1 (Fig. 11) there was again (see Fig. 3) a 90% decrease in FFL activity. This shows that the last 25 bp of the first 100 bp (sequence positions -691 to -667) region of the full length promoter play a crucial role in the regulation of the promoter.

3.6. Bioinformatics analysis of the 25 bp (core promoter region wdr16CPR)

To search for the potential TF binding site in the core promoter region (last 25 bp of the first 100 bp region of the full length promoter, i.e., sequence positions -691 to -667), the 25 bp were analysed with the ConSite program. The site for SOX5 was the only TF binding site found in the range of these 25 bp. It has recently been found that the sox5S SRY-related high mobility group TF was identified as crucial in the transcriptional regulation of sperm-associated antigen 6 (SPAG6; Kiselak et al., 2010).

3.7. Analysis of the transcription factor binding site in the core promoter region wdr16CPR by deletion analysis

Since the deletion of the core promoter region wdr16CPR (last 25 bp of the first 100 bp region of the full length wdr16 promoter, i.e., sequence positions -691 to -667) caused a 90% decrease in the FFL activity, further search for the TFBS was done by deletion analysis. In comparison to EPCs infected with the viral vector CTRL (Fig. 12), there was no decrease in the FFL activity for the EPCs infected with viral vectors C1C1 (Fig. 13), C1C2 (Fig. 14) or C1C3 (Fig. 15), whereas in EPCs treated with C1C4 (Fig. 16) and C1 (Fig. 17), the FFL activity was decreased by about 60-70 %. The deletion of the last 10 bp, i.e., sequence positions -676 to -667 of the 25 bp region reduced the promoter strength by about 65%. The corresponding DNA sequences, especially the sox5 TFBS identified therein, offer themselves for further analysis concerning functional binding of TF Sox5 to the core promoter region.

3.8. Co-transfection of EPCs with wdr16 promoter and sox5

Bioinformatic analysis of the core promoter region wdr16CPR and analysis of the TFBS showed that Sox5 has its binding site in the core promoter region. Therefore sox5S and sox5L were individually cloned under the control of the EF1α promoter and they were cotransfected with CTRL viral vector on EPCs. The EPCs cotransfected with sox5L and CTRL (Fig. 20) showed a 30% increase in the FFL activity in comparison with the EPCs cotransfected with sox5S and CTRL (Fig. 19). This shows that sox5L could play a role in the regulation of wdr16. The molecular mechanism underlying the role of sox5L in transcription is poorly understood. The col2a1 gene is the only one for which strong data, obtained both in vitro and in vivo, support the hypothesis that it might be a direct target of sox5L (Lefebvre et al., 2001). However, to definitely test this hypothesis, evidence still needs to be provided that Sox5 does bind to the Col2a1 enhancer in vivo and that this enhancer directs the expression of col2a1 in vivo. The present work appears to provide the first evidence for showing the involvement of Sox5L in the kinocilia specific gene expression.

3.9 Analysis of the expression level of wdr16 in EPCs co-transfected with wdr16 promoter and sox5

In order to see if there is an increase in the level of Wdr16 expression in EPCs cotransfected with the CTRL vector and sox5S or sox5L, Western blot analysis was carried out. The blots (Fig. 25) showed an about 30% increase in the level of wdr16 expression on the cultures cotransfected with CTRL and Sox5L in comparison to the cultures cotransfected with CTRL and Sox5S (Table 2). This result supports the results obtained from the luciferase assay (Fig. 19 and Fig. 20) where there was a 30% increase in the FFL activity in the EPCs cotransfected with the viral vectors CTRL and sox5L.

3.10 Electrophoretic mobility shift assay (EMSA)

In order to demonstrate that the TF Sox5 binds with the core promoter region wdr16CPR (last 25 bp of the first 100 bp of the full length promoter, i.e., sequence positions -691 to -667) EMSA was performed. The result (Fig. 27, lanes 6 and 7) showed that the Wdr16CPR formed a distinct DNA-protein complex with the nuclear extracts from HEK 293T cells transformed with the sox5S or sox5L mammalian expression vector, like in the positive controls.

The signal for the competition test (Fig. 27, lanes 8 and 9) was reduced distinctly which shows the observed signals are specific for sox5 and there was no signal in the negative controls. This result demonstrates that the TF Sox5 is involved in the regulation of Wdr16. All the findings in this thesis helped to show that the TF Sox5 regulates the kinocilia-specific gene wdr16. This also supports the recent finding that Sox5S regulates SPAG6 gene expression (Kiselak et al., 2010). The binding of a transcription factor to its cognate site in a promoter region is a necessary but not a sufficient condition for the regulation of a gene (Pilpel et al., 2001; Gao et al., 2004). The involvement of Sox5L shown in the present thesis may well be the first evidence of Sox5 participation in kinocilia-specific gene expression.

4. Materials and Methods

4.1. Materials

4.1.1. Devices

Autoclaves	1) Type 669, Aigner, München, 2) Fedegari,Spain 3) Type 5075 ELV, Tuttnauer, Systec
Cameras	1) Coolpix 995, Nikon, Düsseldorf 2) Canon EOS 350D, Canon, Krefeld
Cell incubators	Types B 5060 EC CO_2 and Function LINE, Heraeus, Hanau
Centrifuges	1) Varifuge K, Heraeus, Hanau 2) Biofuge Fresco, Heraeus, Hanau 3) Multifuge 3 S-R, Heraeus, Hanau
Containment hoods	1) Lamin Air HLB 2448, TL 2448, Heraeus, Hanau 2) Technoflow 3F150-11GS, Integra Biosciences, Zizers,Switzerland
Cryosystem	Chronos 80 and liquid N_2 tank Jupiter, Colora E-80, Lorch, Cryotherm, Kirchen/Sieg
Drying oven	Type U-30, Memmert, Schwabach
Electroblotting chamber	Semi-Dry Blotting apparatus, Bio-Rad, München
Electrophoresis chambers	1) For agarose gels: Model B1 (110 mm x 90 mm), Owl separation systems, Asheville, USA 2) For polyacrylamide gels: Wide Mini-Sub Cell GT System, Bio-Rad, München
Gel documentation system	Molecular Imager, Mitsubishi, Osaka, Japan
Heating block	Grant QBT, CLF Laborgeräte, Emersacker
Hybridisation oven	Biometra, Göttingen
Magnetic stirrer	IKAMAG RCT, Bachofer, Reutlingen
Microplate reader	Titertec Plus MS 212, Meckenheim Sofware used: ICN, Costa Mesa, California, USA

Microscopes	1) Models IM, IM 35 and Axiovision 2, Zeiss, Oberkochen 2) Axiovert 25, Leitz, Oberkochen
Osmometer	Osmometer Automatic, Knauer, Eppelheim
PCR Thermocycler	Primus 96 plus, MWG AG Biotech, Ebersberg
pH meter	PHM 92, Radiometer, Copenhagen, Denmark
Pipettes	1) Finn pipettes (5-40 µl, 40-200 µl, 200-1,000 µl), Thermo-Labsystems, Vantaa, Finland 2) Eppendorf pipettes (0.5-10 µl, 10-100 µl, 200-1,000 µl), Eppendorf, Hamburg 3) Multichannel pipette, Titerman, Eppendorf, Hamburg
Power supplies	1) Consort E 132, BioBlock Scientific, Illkirch, France 2) 2301 Macrodrive 1, LKB Bromma, Vienna, Austria 3) Power Supply Model 200, Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD, USA 4) Computer Controlled Electrophoresis Power Supply Model 3000 X, Bio Rad, München
Rotor for Ultracentrifugation	Type 70 Ti, Beckman-Coulter, Brea, California, USA
Balances	Types 1403 and L2205, Sartorius, Göttingen
SDS-PAGE device	Ready Gel Cell, Bio-Rad, München
Shaker	Vortex Genie, Bender & Hobein, München
Shaking platform	Horizontal-Schüttelplattform KL2, Bühler, Tübingen
Sonifier	Branson B-30 with microtip, Heinemann, Schwäbisch Gmünd
Spectrophotometers	Uvikon 860 with Plotter 800, Kontron, Eching
Tabletop centrifuge	Centrifuge 5415 C, Eppendorf, Hamburg
Tissue homogenizer	Potter-Elvehjem homogeniser, Braun, Melsungen
Ultracentrifuge	Optima L-80, Beckman-Coulter, Brea, California, USA
UV transilluminator	UVP, San Gabriel, California, USA

Water baths	1) Julabo Standard, Julabo PC Thermostat, Labora, Mannheim 2) GFL-1083 shaking water bath, Helago laboratory equipment, Medingen
Water purification unit	USF Elga (0.22 µm-filter), Purelabs, USA
Welding apparatus for preparing nylon mesh bags	Super Poly 281, Audion Elektron, Kleve
X-ray film developing machine	Röntgenfilm Entwicklungsmaschine SRX-101, Konica Europe, Hohenbrunn
X-ray film exposure cassette	Hypercassette, Amersham Buchler, Braunschweig

4.1.2. General Materials

Coverslips 12 mm (round), 18 mm x 18 mm (square)	Roth, Karlsruhe
Cryo tubes, 2 ml	Greiner, Frickenhausen
Culture dishes, (35 mm in diameter)	Becton Dickinson, Heidelberg
Culture dishes, (90 mm in diameter)	Nunc, Wiesbaden
Culture flasks, 75 cm^2	Nunc, Wiesbaden
Filter paper (Whatman 3 MM)	Whatman, Göttingen
Filtration units (sterile Millex units)	Millipore, Eschborn
Glass pipettes, 1 ml, 5 ml, 10 ml	Hirschmann, Eberstadt
Glass ware	1) Schott, Mainz; 2) Brand, Wertheim
Hybridisation container	Fisher Scientific, Schwerte
Microscope slides (26 mm x 76 mm x 1 mm)	Menzel via Roth, Karlsruhe
Microscope slides “Superfrost” (26 mm x 76 mm x 1 mm)	Menzel via Roth, Karlsruhe

Microtiter plates (Maxisorp Immuno plate F96)	Nunc, Wiesbaden
MµltiGuard ™ barrier tips	Sorenson Biosciences, Inc., via Roth, Karlsruhe
Nitrocellulose membrane (Trans-Blot, 0.45 µm)	Bio-Rad, München
Nylon cloth (132 and 210 µm mesh size)	Sefar GmbH, Wasserburg/Inn
Nylon membrane, positively charged	QBIOgene, Heidelberg
PCR tubes (0.2 ml)	PeqLab, Erlangen
Petri dishes (AD94/H16mm)	Roth, Karlsruhe
Pipette tips	Braun, Melsungen
Plastic centrifuge tubes, 14 ml	Greiner, Frickenhausen
Plastic centrifuge tubes, 50 ml	Nunc, Wiesbaden
Plastic reaction tubes, 1.5 ml	Brand, Wertheim
Pursept®-A disinfectant solution	Merz Hygine GmbH, Frankfurt
Safe Skin Satin Plus powder-free latex gloves	Kimberly Clark, Koblenz-Rheinhafen
Sterile filters (0.2 µm and 0.45 µm)	Renner GmbH, Dannstadt
Sterile single use serological pipets (10 ml)	Falcon, Kirchheim/Teck
Syringes, 20 ml, sterile	Braun, Melsungen
X-ray film	Amersham, Freiburg

4.1.3. Chemicals

3-Amino-1,2,4-triazole	Sigma-Aldrich, Steinheim
APS	Fluka, Steinheim
Bradford assay dye reagent	Bio-Rad, München
Bromophenol blue	Fluka, Steinheim
BSA	Sigma-Aldrich, Steinheim
$CaCl_2 \cdot 2H_20$	E. Merck, Darmstadt
"CDP-Star"chemiluminescence substrate	Roche, Mannheim
Coomassie brilliant blue R 250	Sigma-Aldrich, Steinheim
D-Glucose	Fluka, Steinheim
Dithiothreitol (DTT), 0.1 M	Gibco BRL, Karlsruhe
DMSO	Sigma-Aldrich, Steinheim
"Empigen"detergent 30% (w/w)solution	Calbiochem, Darmstadt
Ethylenediaminetetraacetic acid	Roth, Karlsruhe
FuGENE®6	Roche, Mannheim
Glycerol	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
"Immu-mount"mounting medium	Thermo Shadon, Pittsburgh, PA, USA, via Thermo Electron, Bremen
JetPEI™ transfection reagent	QBIOgene, Heidelberg
Mercaptoethanol	Roth, Karlsruhe
$MnCl_2$	Fluka, Steinheim
3-(N-Morpholino)propanesulfonic acid (MOPS)	Fluka, Steinheim
NaCl	Roth, Karlsruhe
$NaHCO_3$	Roth, Karlsruhe
p-Nitrophenylphosphate	Roche, Mannheim
PageRuler™ prestained protein ladder	MBI Fermentas, St. Leon-Rot
Paraformaldehyde	Fluka, Steinheim
KCl	E. Merck, Darmstadt
RbCl	Sigma-Aldrich, Steinheim

Roti®-Block 10x Concentrate	Roth, Karlsruhe
Roti®-ImmunoBlock 10x Concentrate	Roth, Karlsruhe
Rotiphorese® Gel 30	Roth, Karlsruhe
Sodium hypochlorite solution	Roth, Karlsruhe
SDS	Fluka, Steinheim
Saccharose	Roth, Karlsruhe
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Steinheim
Tris(hydroxymethyl)aminomethane	Roth, Karlsruhe
Tween® 20	Fluka, Steinheim

4.1.4. Kits

Advantage® 2 PCR kit	Clontech (now Takara Bio), Heidelberg
DNeasy® Tissue kit	Qiagen, Hilden
Enhanced chemiluminescence (ECL) detection reagent	Amersham, Freiburg
HotStarTaq Master Mix kit	Qiagen, Hilden
Match maker one hybrid library construction and screening kit containing, among others, T7 primer and Amplimer	Clontech (now Takara Bio), Heidelberg
NucleoSpin® Extract II kit	Macherey-Nagel, Düren
NucleoSpin® Plasmid kit	Macherey-Nagel, Düren
NucleoSpin® Xtra Midi Plus kit	Macherey-Nagel, Düren
NucleoSpin ® mini prep Kit	Macherey-Nagel, Düren
Omniscript Reverse Transcriptase kit	Qiagen, Hilden
QIAGEN PCR Cloning^{plus} kit	Qiagen, Hilden
QIAprep spin miniprep kit	Qiagen, Hilden
QIAquick PCR purification kit	Qiagen, Hilden
RNeasy RNA isolation kit	Qiagen, Hilden
GSL Assay kit	NEB, Frankfurt, Germany
Bright-Glo Kits	Promega, Mannheim, Germany

4.1.5. Reagents for Molecular Biology

Agarose	PeqLab, Erlangen
GeneRuler 1 kb DNA ladder	MBI Fermentas, St. Leon-Rot
GeneRuler 100 bp DNA ladder plus	MBI Fermentas, St. Leon-Rot
Deoxyribonucleoside triphosphates	PeqLab, Erlangen
Ethidium bromide	Fluka, Steinheim
Oligo(dT_{15}) primer	Invitrogen, Karlsruhe
PCR primers	Invitrogen, Karlsruhe; Biomers, Ulm
Phenol/chlorophorm/isoamylalcohol (25 :24 :1) (v/v/v)	Applichem, Darmstadt
RNAse inhibitor (40 U/µl)	Promega, Mannheim
T4 DNA Ligation Buffer (10x)	MBI Fermentas, St. Leon-Rot

4.1.6. Enzymes for MolecularBiology

BamHI	New England Biolabs, Frankfurt a.M.
Calf intestine alkaline phosphatase (1U/µl)	MBI Fermentas, St. Leon-Rot
ClaI	New England Biolabs, Frankfurt a.M.
EcoRI	New England Biolabs, Frankfurt a.M.
EcoRV	New England Biolabs, Frankfurt a.M.
HindIII	New England Biolabs, Frankfurt a.M.
HotStart Taq DNA polymerase	Qiagen, Hilden
MfeI	New England Biolabs, Frankfurt a.M.
MluI	MBI Fermentas, St. Leon-Rot
Omniscript Reverse Transcriptase	Qiagen, Hilden
PacI	New England Biolabs, Frankfurt a.M.
PfuUltra™ Hotstart High-Fidelity DNA polymerase	Stratagene, Amsterdam
Polynucleotide kinase	MBI Fermentas, St. Leon-Rot
PstI	New England Biolabs, Frankfurt a.M.
PvuII	New England Biolabs, Frankfurt a.M.
RNAse A	Qiagen, Hilden

RsrII	MBI Fermantas, St.Leon-Rot
SalI	MBI Fermentas, St. Leon-Rot
SplI	MBI Fermantas, St.Leon- Rot
Taq polymerase	Eppendorf, Hamburg
T4 DNA ligase	MBI Fermentas, St. Leon-Rot
“TripleMaster”polymerase mix (5U/µl)	Eppendorf, Hamburg
XhoI	New England Biolabs, Frankfurt a.M.

4.1.7. Constituents and reagents for bacterial and mammalian cell cultures

Antimycotic/antibiotic stock solution	Sigma-Aldrich, Steinheim
Carbenicillin, disodium salt	Roth, Karlsruhe
DMEM, powder medium lacking pyruvate and $NaHCO_3$	GibcoBRL, Karlsruhe
FCS	Biochrome, Berlin
HBSS	GibcoBRL, Karlsruhe
Insulin	Sigma-Aldrich, Steinheim
Kanamycin	Applichem, Darmstadt
LB-agar powder	Fluka, Steinheim
LB-broth powder	Fluka, Steinheim
MEM powder	GibcoBRL, Karlsruhe
Penicillin G, potassium salt	Serva, Heidelberg
Superoptimal broth, catabolite repression (SOC) medium	Novagen, Schwalbach/Ts.
Streptomycin sulfate	Serva, Heidelberg
Thrombin (human)	Provided by Dr. Mirna Rapp, Aventis Behring, Marburg
Transferrin	Roche, Mannheim
Trypsin	ICN, Eschwege

4.1.8 . Antibodies

4.1.8.1. Primary antibodies

Monoclonal anti-acetylated α-tubulin antibody	Sigma-Aldrich, Steinheim
Monoclonal anti-β-actin antibody	Sigma-Aldrich, Steinheim
Polyclonal antibody against HIV-1 p24	Acris, Herford
Polyclonal antibody against HIV-1 p24 (biotin)	Acris, Herford
Anti-Wdr16 serum (from rabbit)	Provided by Dr. Wolfgang Hirschner

4.1.8.2. Reagents for detection of primary antibodies

Donkey anti-guinea pig IgG Cy3 conjugate	Jackson, via Dianova, Hamburg
Donkey anti-guinea pig IgG peroxidase conjugate	Jackson, via Dianova, Hamburg
Goat anti-mouse IgG Alexa Fluor 568 conjugate	Molecular Probes, via Invitrogen, Karlsruhe
Goat anti-mouse IgG peroxidase conjugate	Jackson, via Dianova, Hamburg
Donkey anti-rabbit IgG peroxidise conjugate	Jackson, via Dianova, Hamburg
Goat anti-rabbit IgG alkaline phosphatase conjugate	Jackson, via Dianova, Hamburg

4.1.9. Bacterial strains

DH5α E.coli cells	Gift from Dr. Frank Madeo, Graz, Austria
NovaBlue Singles™ competent cells	Novagen, Schwalbach/Ts.
Qiagen EZ competent cells	Qiagen, Hilden

4.1.10. Mammalian cell line

293T Human embryonic kidney cells	Provided by Dr. Roland Vogel, Paris

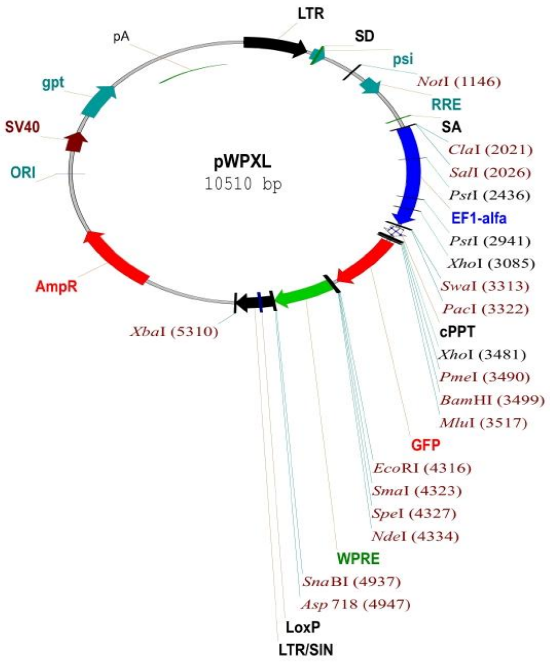
4.1.11. Animals

Wistar rats	Purchased from Charles River, Kisslegg; bred in the animal facility of the institute

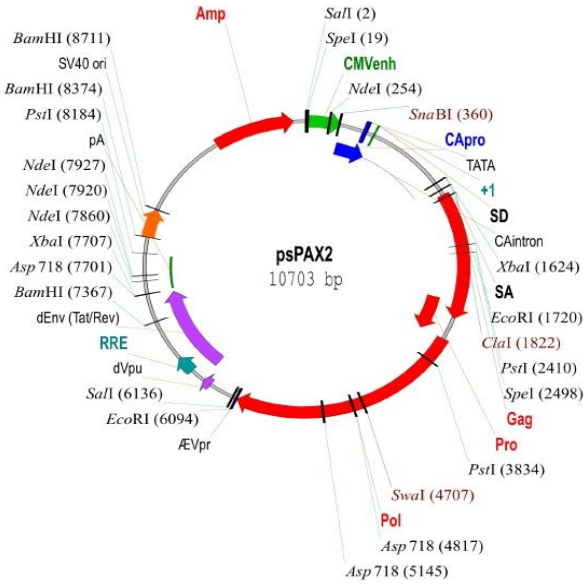
4.1.12. Vectors

4.1.12.1. Lentiviral Vectors

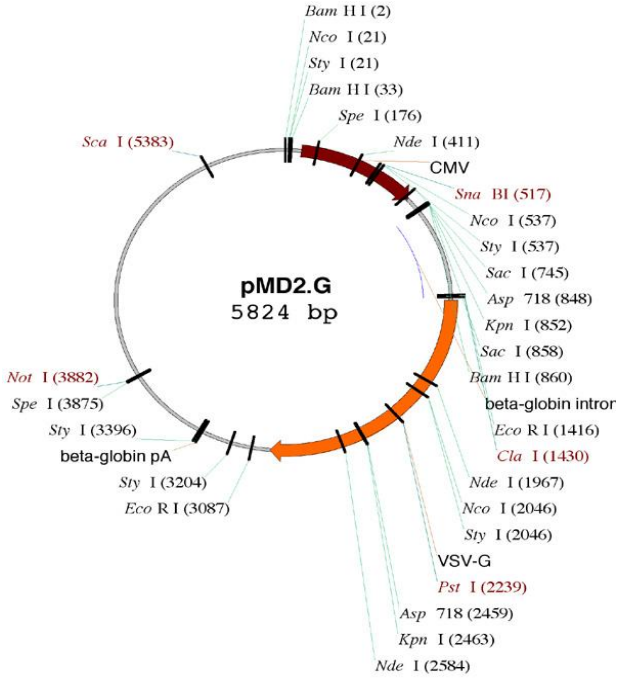
pWPXL



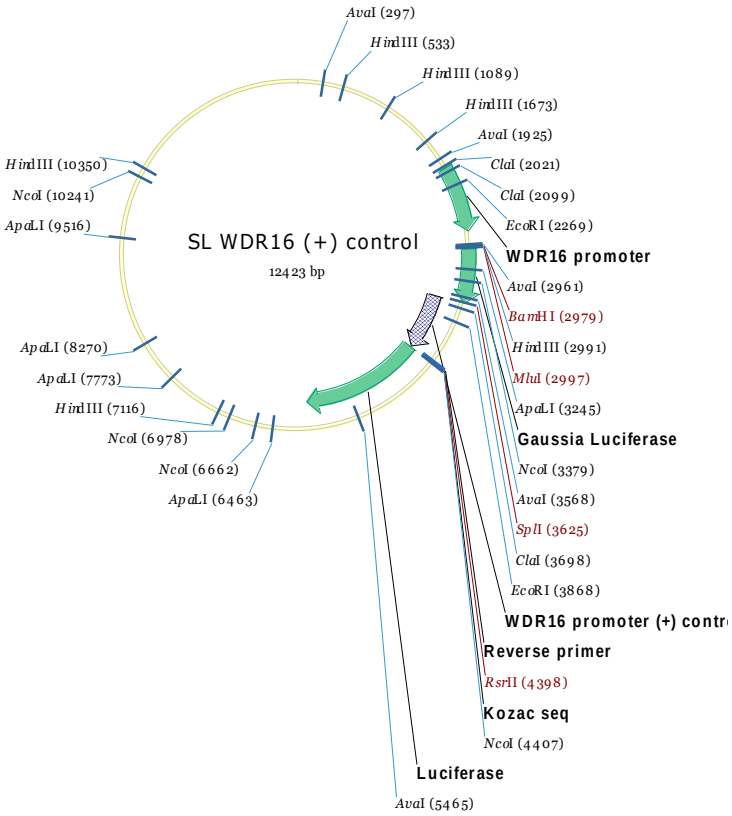
psPAX-2



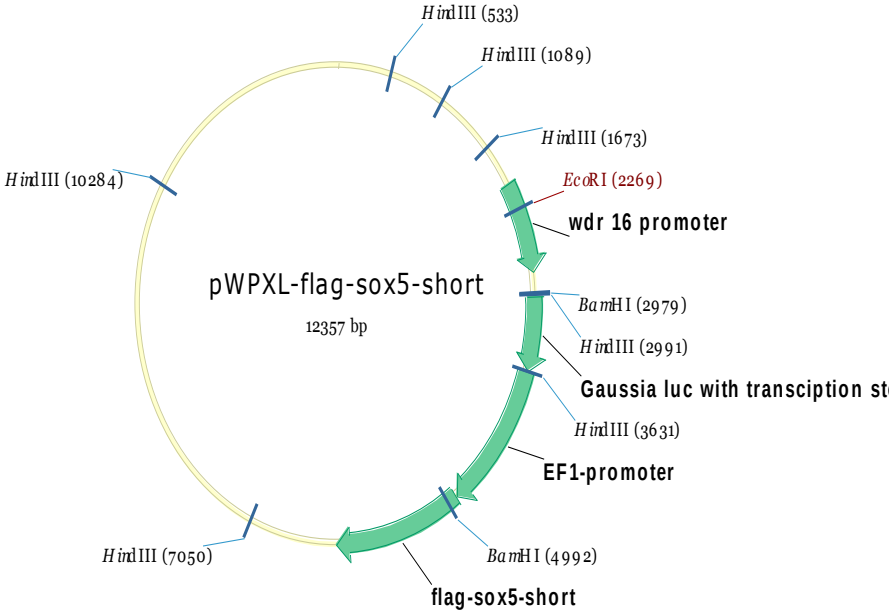
pMD2.G



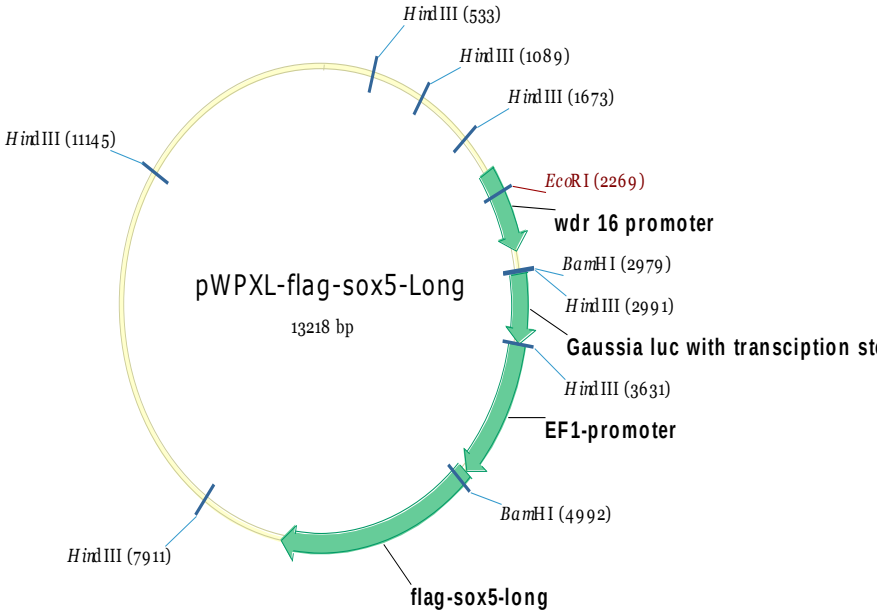
CTRL Vector 2



pWPXL- sox5S

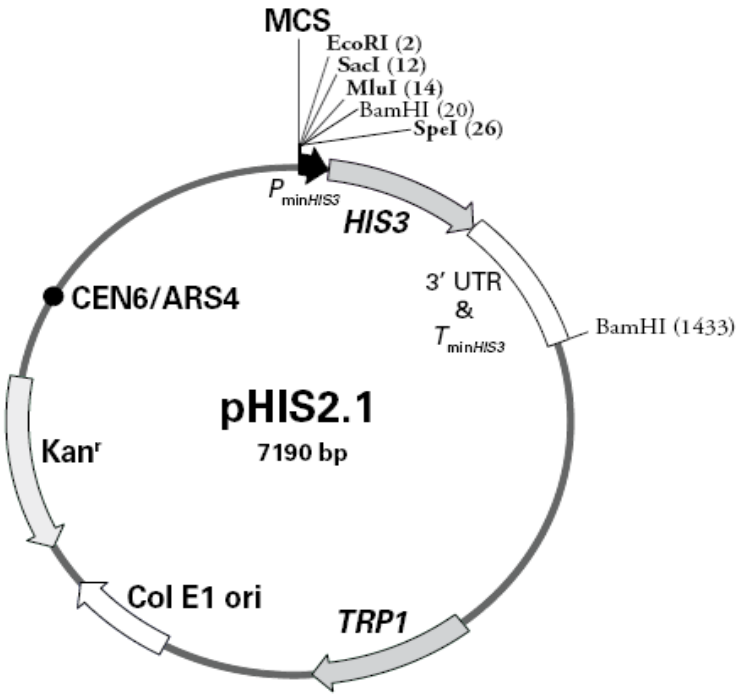


pWPXL – sox5L

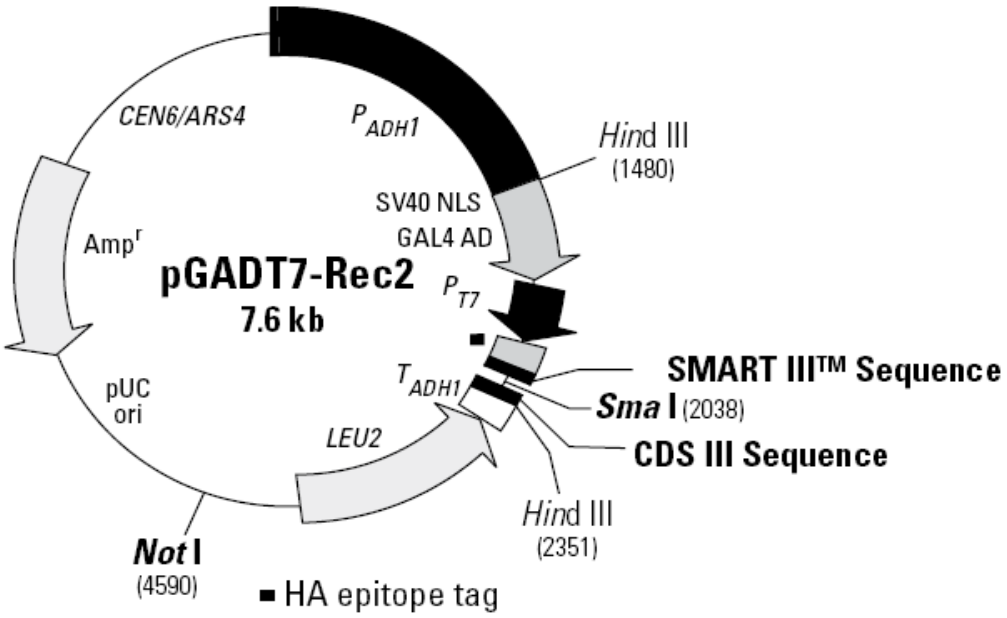


4.1.12.2. Vectors used for Yeast one hybrid assay

pHIS2.1 - Clontech (now Takara Bio), Heidelberg



pGADT7-Rec2



4.2. Methods

4.2.1. Cell culture

4.2.1.1. Media and solutions for cell culture

Penicillin/streptomycin (PS) stock solution

651 mg penicillin G, potassium salt (1536 U/mg) and 1 g streptomycin sulfate (750 U/mg), dissolved in 50 ml ddH_2O

Puck's D1 solution

137 mM NaCl, 5.4 mM KCl, 0.22 mM KH_2PO_4, 0.17 mM Na_2HPO_4; pH 7.4

Puck's D1 / Gluc / Suc solution

5 mM D-glucose, 58.4 mM sucrose, in Puck`s D1 solution

Trypsin solution 0.05 % (w/v)

Puck's D1 / Gluc / Suc solution supplemented with 0.2% (w/v) phenol red and 0.05% (w/v) trypsin

MEM_{Wash}

9.65 g MEM powder and 2.2 g $NaHCO_3$, dissolved in 1 l ddH_2O. The medium was gassed with CO_2 until the colour became orange.

MEM_k

MEM_{wash} medium supplemented with 0.5 g/l BSA, 5 mg/l insulin, 10 mg/l transferrin and 1 ml PS stock solution per liter of medium

MEM_{kT}

MEM_k supplemented with 500 U/l thrombin

DMEM

133.75 g DMEM powder without pyruvate and $NaHCO_3$, 2.2 g sodium pyruvate and 74 g $NaHCO_3$ were dissolved in 20 l ddH_2O. The medium was gassed with CO_2 until the colour became orange. The osmolarity was 320-340 mOsmol/l.

90% DMEM / 10% FCS

900 ml DMEM plus 100 ml FCS

90% DMEM / 10% FCS / PS

1 ml PS stock solution was added to 1 l 90% DMEM / 10% FCS

90% DMEM / 10% FCS / antimycotic

1 ml 1,000-fold concentrated antimycotic stock solution (see page 62) was added to 1 l 90% DMEM / 10% FCS

4.2.1.2. Ependymal primary cultures (EPC)

EPC were prepared from the brains of newborn Wistar rats or BL6 mice not older than 24 h according to the method of Weibel et al. (1986) and as modified by Prothmann et al. (2001).

Coating of culture dishes with fibronectin

Fibronectin was isolated according to Miekka et al. (1982) with modifications described by Prothmann et al. (2001). Culture dishes either 35 mm in diameter or wells of 24-well plates with round coverslips (12 mm in diameter) were incubated at 37°C with 0.7 ml or 200 µl of filter-sterilised fibronectin solution (200 µg/ml), respectively, for 2 h. After incubation, the fibronectin solution was removed and the culture dishes were refilled with 2 ml MEM_{wash}. Coated culture dishes were incubated at 37°C in an incubator with an atmosphere of 95% air and 5% CO_2 until cells were seeded.

Preparation of rat / mouse brains

Newborn rats / mice were decapitated with a pair of scissors. The brain was squeezed through the foramen magnum by application of pressure to the roof of the skull. It was then transferred to a sterile Petri dish 90 mm in diameter containing 10 ml ice-cold Puck‘s D1 / Gluc / Suc solution.

Dissociation of rodent brains into single cells

Prior to the procedure, nylon gauze bags were prepared using nylon cloth (132 µm and 210 µm mesh size) with a welding apparatus. These nylon bags were autoclaved before they were used for the preparation of the cultures. The brains were packed into a 210 µm mesh size nylon gauze bag and dissociated by massaging them through the mesh with sterile forceps. The ensuing suspension was collected in a Petri dish filled with 10 ml ice cold Puck's D1 / Gluc / Suc solution, where it was triturated to essentially apparent homogeneity with a 10 ml glass pipette. Remaining large aggregates were further removed by passing the cell suspension through a 132 µm mesh size gauze bag, and the cell suspension was collected into a 50 ml plastic centrifuge tube. This tube was centrifuged in a Heraeus Varifuge K centrifuge at 4°C and 1500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 10 ml of MEM_k. This suspension was passed through a 132 µm mesh size gauze bag into an appropriate volume of MEM_k to yield the seeding suspension. For each dissociated brain, 30 ml of MEM_k were used, as described in Weibel et al. (1986).

Seeding of dissociated brain cells

MEM_{wash} was removed from fibronectin-coated dishes and replaced by either 2 ml or 0.5 ml MEM_{wash}, respectively, for dishes of 35 mm diameter and and wells of 24-well plates. Seeded cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. After 2 days, MEM_k was replaced by MEM_{kT}. The culture medium was renewed every 2 days. The viability and the quality of the cultures were regularly monitored under a phase-contrast microscope. Appearance of rolled up carpets of ependymal cells are indicative of undesirable cell condition barring use for any kind of further analysis.

4.2.1.3 Cultures of the human embryonic kidney cell line HEK293T

Starting the culture

The HEK293T cell line was kindly provided by Dr. Roland Vogel as a cryo stock at passage number 5. To take the cells into culture, the cryo stock was rapidly thawed at 37°C in a pre-warmed circulating water bath. Then the cell suspension was transferred to a 25 cm^2 culture

flask containing 20 ml 90% DMEM /10% FCS /PS and subsequently incubated at 37°C in an atmosphere of 90% air and 10% CO_2 for 4 h. After attachment of the cells to the culture dish (approximately 4 h after seeding), the medium was replaced by fresh DMEM / FCS supplemented with antimycotic (amphotericin B) and antibiotic (penicillin/streptomycin).

Maintenance of the cell culture

The HEK293T cell line was cultured in 25 cm^2 culture flask using DMEM / FCS at 37°C in humidified atmosphere containing 10% CO_2 The medium was renewed every third day. The degree of confluency and the quality of the cultures were routinely monitored under an inverted phase contrast microscope.

Passaging of the cells

When the cells had reached confluency in the 25 cm^2 flask (after approximately 4-5 d), the culture medium was removed and adherent cells were washed with 1 ml 0.05% trypsin solution. After discarding the washing solution, 0.05% trypsin solution (2 ml) was added and the flask was incubated at 37°C until cells were detached (approximately 2-3 min). To inhibit trypsin activity, detached cells were resuspended in 10 ml DMEM/FCS/PS and subsequently transferred to a 50 ml centrifuge tube. After centrifugation (1,000 rpm / 4°C / 5 min) in a Heraeus Multifuge 3 S-R, the supernatant was discarded and the cell pellet was triturated with 15 ml DMEM / FCS, using a disposable 10 ml pipette. To start a new passage, 500 µl of this cell suspension was added to a new culture flask containing 10 ml DMEM / FCS.

Preparation of cryo stocks

Freezing medium: 80% DMEM + 15% FCS + 5% DMSO; filter-sterilized and stored at -20°C.
For the preparation of cryo stocks, cells of 80-90% confluent cultures were detached with 0.05% trypsin (5 min / 37 °C), then collected by centrifugation (1,000 rpm / 4°C / 5 min) before being resuspended in 3 ml of freezing medium. The suspension was transferred to three 1 ml cryo vials and frozen at -80°C after wrapping with paper towels. Later the cryo stocks were transferred to a cell bank and stored above liquid nitrogen.

4.2.2. Production of lentiviral vectors in HEK293T cells

Solutions

- Calcium chloride: 2.5 M solution in ddH_2O, filter-sterilized
- 2x HBS: (for 500 ml) 8 g NaCl, 0.38 g KCl, 0.1 g Na_2HPO_4, 5 g HEPES, 1 g glucose; pH 7.05, filter sterilized
- MEM_k supplemented with 25 mM glucose

Experimental procedure

Day 1: Plating

The day before transfection, HEK293T cells suspended in 90% DMEM / 10% FCS (v/v) were seeded into cell-culture dishes (90 mm in diameter) at a density of 2.5 – 3.0 million viable cells per plate.

Day 2: Transfection

Transfection was done using the calcium phosphate method (Jordan et al., 1996). Calcium phosphate precipitate (1 ml per culture dish 90 mm in diameter) was prepared as described there. Briefly, 20 µg of transfer vector (pWPXL), 15 µg of packaging plasmid (psPAX2) and 6 µg of envelope plasmid (pMD2.G) were put into a sterile reaction tube the final volume of which amounted to 150 - 200 µl. To this plasmid solution, 50 µl of 2.5 M $CaCl_2$ solution was added. With autoclaved ddH_2O, this suspension was made up to a final volume of 500 µl and then mixed thoroughly (Vortex). This suspension was then carefully and slowly, i.e, drop by drop, added to a tube containing 500 µl of double-concentrated HBS solution and then mixed thoroughly (Vortex). Incubation at RT for 1 min was followed by dropwise addition of the precipitate to the cells under gentle shaking of the plate. The plate was then incubated at 37°C in a humidified atmosphere of 90% air and 10% CO_2. After 6-8 h of transfection, the calcium phosphate precipitate-containing medium was removed and the cells were washed briefly with 5 ml of 90% DMEM / 10% FCS (v/v). Then 6 ml of fresh MEM_k supplemented with 25 mM glucose were added and the plate was incubated at 37°C in a humidified atmosphere of 90% air and 10% CO_2.

Days 3, 4 and 5: Collection of virus

The virus containing supernatant was harvested at day 3 into a sterile tube, centrifuged (3,000 rpm, RT, 5 min), passed through a filter with a pore diameter of 0.45 µm and stored at 4°C. Supernatant was also harvested on days 4 and 5 after transfection, pooled with the first sample and frozen at -80°C for future use. Alternatively, the virus was concentrated by ultracentrifugation (100,000 g, 4°C, 2 h).

For large scale production of lentivirus, 24 cell cultures in dishes 90 mm in diameter were transfected and the virus supernatant was harvested as described above.

4.2.3. Concentration of lentiviral vectors

Virus suspension was transferred into sterile Beckman 25 mm X 89 mm centrifuge tubes and centrifuged (100,000 g, 4°C, 2 h) in an Optima L-80 Ultracentrifuge (Beckman-Coulter) using a Type 70 Ti rotor. After the spin, the supernatant was discarded and the viral pellet was resuspended in 1 ml of MEM_k. Aliquots of 40 µl were stored at -80°C for future use.

4.2.4. Lentiviral vector mediated gene transfer

EPCs were infected with lentiviral vectors either on DIV 1 or on DIV 6. During the infection on DIV 1, MEM_k was used for dilution of the viral stock, while it was MEM_{kT} on DIV 6. While infecting with the non-concentrated virus, care was taken that the volume of virus stock never exceeded the volume of medium above the cells. In case of the concentrated viral stocks, 200 ng of HIV-1 p24 per well 15 mm in diameter or an MOI of 40 was used as a maximal virus load. The total volume of medium per well during infection was 250 µl, which was doubled to 500 µl when the virus-containing medium was replaced by fresh medium during an overnight incubation. The transfected EPCs were maintained with regular (every second day) media changes at 37°C in a water vapour-saturated atmosphere of 5% CO_2 and 95% air until they were used for further analysis.

4.2.5. Preparation of medium for bacterial cultures

Carbencillin stock solution in ddH_2O: 50 mg/ml H_2O (1,000 fold concentrated)
Luria broth (LB; 2%): Was prepared, steam sterilized and allowed to cool to approximately 50^oC. Then 1 ml of 1,000 fold concentrated carbencillin stock solution was added. The ensuing mixture was stored at 4 ^oC.

Preparation of agar plates

LB agar (1 l, 3%) was prepared, steam sterilized and allowed to cool to 50^oC. Then 1 ml of 1,000 fold concentrated carbenicillin solution was added. The solution was aseptically poured into Petri dishes (approximately 10 ml/dish). After solidification of the agar the plates were stored at 4 ^oC.

Bacterial liquid cultures

A single bacterial colony from the agar plate was transferred to 4 ml of LB medium with antibiotic (carbenicillin) under aseptic conditions. The liquid cultures were incubated overnight at 37^oC on a shaker. This was done to amplify the single bacterial colony for plasmid isolation.

Preparation of bacterial glycerol stocks

Bacterial stocks were prepared by mixing 0.5 ml of the overnight culture of the bacteria with 0.5 ml of glycerol in cryotubes and frozen at -80 ^oC.

4.2.5.1. “Mini” scale preparation of plasmid DNA from bacterial liquid cultures

An overnight bacterial culture (4 ml) was centrifuged (3,000 rpm /4°C /5 min) using a Heraeus centrifuge (table top centrifuge). The supernatant was discarded. Plasmid DNA was isolated from the pellet using a NucleoSpin® Plasmid miniprep kit (Macherey-Nagel) following the manufacturer’s instructions.

Briefly, the bacterial pellet was resuspended in 250 µl of buffer A1 supplemented with RNaseA (200 µg/250 µl). Then an equal volume of buffer A2 was added. The contents of the vessel were mixed gently by inverting the tube 6-8 times. This was followed by incubation at RT for approximately 5 min. Thereafter, 300 µl of buffer A3 was added. The conents of the tube were mixed by inverting the tube 6-8 times. Then the bacterial lysate was centrifuged (13,000 rpm, RT, 10 min) in a table top centrifuge. After centrifugation, the supernatant was transferred to a NucleoSpin® Plasmid column supplied with the collection tube. After another centrifugation (13,000 rpm, RT, 1 min), the flowthrough collected was discarded and the silica column was washed with 600 µl of buffer A4 (containing ethanol) by centrifugation (13,000 rpm, RT, 1 min). The silica column was dried again by brief centrifugation (13,000 rpm, RT, 1 min). After this, the column was placed in a sterile 1.5 ml microfuge tube and the plasmid DNA was eluted with 50 µl of buffer AE by centrifugation (13,000 rpm, RT, 1 min). The isolated DNA was quantified photometrically (see section 4.2.5.6).

4.2.5.2. Isolation of total cellular RNA from cultured cells

Total cellular RNA was isolated using an RNeasy RNA isolation kit (Qiagen). Briefly, approximately 10^7 cells (HEK 293 and EPCs) were detached by trypsinisation and a cell pellet was obtained by brief centrifugation (2,000 rpm, RT, 5 min). To the cell pellet 600 µl of buffer RLT were added and the cells of the pellet were suspended by repeated pipetting. An equal volume of 70% ethanol was added to the lysate and the suspension was mixed well by pipetting. This suspension was transferred to an RNeasy spin column supplied with the collection tube and centrifuged (13,000 rpm, RT, 15 s). The flow-through was discarded and the column was washed by the addition of 700 µl of buffer RW1 followed by a brief centrifugation (13,000 rpm, RT, 15 s). The flow-through was discarded and the column was again washed with 500 µl buffer RPE (with ethanol) by centrifugation (13,000 rpm, RT, 1 min). To prevent ethanol carry-over and to dry the column, one more centrifugation (13,000 rpm, RT, 1 min) was carried out. After this the column was placed in a sterile 1.5 ml microfuge tube, and the total RNA was eluted with 50 µl of RNase-free water by centrifugation (13,000 rpm, RT, 1 min). The isolated RNA was quantified photometrically (see section 4.2.5.6).

4.2.5.3. Isolation of genomic DNA from cultured cells

Genomic DNA was isolated using the DNeasy tissue kit from Qiagen. To this end, HEK293T cells (approximately 5 x 10^6 viable cells) were detached by trypsinisation and a cell pellet was obtained by brief centrifugation (2,000 rpm, RT, 5 min). The cells of the pellet were resuspended in 200 µl of PBS using a 100-1,000 µl micropipette. The resuspended cells were disrupted by addition of 200 µl buffer AL and 20 µl of proteinase K and incubation at 56°C for 10 min. Thereafter 200 µl of ethanol were added and the contents of the vessel were mixed by repeated pipetting. The suspension was transferred into a DNeasy mini spin column with collection tube, and centrifuged (8,000 rpm, RT, 1 min). The flow-through was discarded and 500 µl of buffer AW1 were added to the column. After another centrifugation (8,000 rpm, RT, 1 min), the flow-through was discarded again and buffer AW2 (500 µl) was added. The column was again centrifuged (13,000 rpm, RT, 3 min) and the flow-through was discarded. The column was dried by another brief centrifugation. The genomic DNA was eluted from the column into a sterile 1.5 ml microfuge tube by the addition of 200 µl buffer AE (10 mM Tris/HCl, 0.5 mM EDTA, pH 9.0) and centrifugation (8,000 rpm, RT, 1 min). The isolated DNA was quantified photometrically (see section 4.2.5.6).

4.2.5.4. Extraction of DNA from agarose gels

DNA samples were loaded in duplicates in adjacent lanes of an agarose gel. The lane placed next to a DNA standard, named the analytical lane, was loaded with a lower volume than the preparative lane from which the DNA was to be extracted later on. After gel electrophoresis, the part of the gel containing the marker lane together with the analytical lane was cut out with a scalpel and stained with 0.005% ethidium bromide / TAE for 10 min and washed several times with water for removing excess stain. The desired band in the analytical lane was cut out under UV illumination and then the analytical lane was again aligned precisely to the preparative lane of the gel in order to secure the correct excision of the desired band from the preparative lane. DNA from this agarose fragment was extracted using a Nucleospin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions.

4.2.5.5. Direct purification of PCR products

The NucleoSpin® Extract II kit (Macherey-Nagel) was used to purify PCR products. Briefly, 2 volumes of buffer NT was mixed with 1 volume of sample (2 ml buffer/ 1 mg of gel) and transferred to a NucleoSpin®Extract II column with collection tube. After centrifugation (13,000 rpm, RT, 1 min), the flow-through was discarded and the column was washed once with 600 µl of buffer NT3. After another centrifugation (13,000 rpm, RT, 1 min) the PCR products were collected in a sterile 1.5 ml microfuge tube by addition of 15-20 µl of elution buffer NE (5 mM Tris/HCl, pH 8.5) and centrifugation (13,000 rpm, RT, 1 min). The isolated DNA was quantified photometrically (see section 4.2.5.6).

4.2.5.6. Photometric determination of nucleic acid concentration

The concentrations of DNA or RNA were determined photometrically by measuring the absorbances (OD) at 260 nm and 280 nm (Warburg and Christian, 1941). Dd H_20 used for diluting the nucleic acids served as the reference (blank). An OD_{260} value of 1 corresponded to a concentration of 50 µg/ml for double-stranded DNA and to 40 µg/ml for RNA (Gallagher and Desjardins, 2007). The concentrations of the DNA or RNA were calculated accordingly. The diluted solutions (1 µl of DNA/RNA in 9 µl of ddH_2O) were prepared in such a way that the absorbance fell into the range from 0.1 to 1, assuring proportionality between nucleic acid concentration and absorbance. Also the purity of samples can be identified using the OD_{260}/OD_{280} ratio. A protein-free nucleic acid solution should have an OD_{260}/OD_{280} ratio between 1.8 to 2.0. (Warburg and Christian, 1941).

4.2.5.7. Dephosphorylation of vector DNA

Dephosphorylation buffer: 500 mM Tris/HCl, 1 mM EDTA; pH 8.0. To the 50 µl of the reaction mixture for digestion with restriction enzyme, 5 µl dephosphorylation buffer and 1 µl CIAP (1 U/µl) were added. The new mixture was incubated at 37°C for 1 h. Thereafter the enzyme was subjected to heat-denaturation (85°C, 15 min)

4.2.5.8. Agarose gel electrophoresis

Solutions

- 50-fold concentrated TAE: 2 M Tris, 0.1 M EDTA, Na salt; pH 8.5 (pH adjusted with glacial acetic acid)
- 5-fold concentrated DNA loading buffer: 40% (w/v) saccharose, 0.25% (w/v) bromophenol blue

Experimental procedure

Agarose gel electrophoresis was used for analytical purposes as well as for preparative DNA purification. Agarose concentrations varied between 1.0% and 1.2%, depending on the expected size of the DNA fragments. Agarose was dissolved in 100 ml of TAE by heating in a microwave oven. The solution was allowed to cool to approximately 50°C. The gel was prepared using a casting chamber of 110 mm x 90 mm. The samples were mixed (5:1) with 5-fold concentrated DNA loading buffer. The samples were electrophoresed at 100 V in TAE buffer (550 ml) for approximately 1.5 h. “Fermentas 100 bp Plus DNA ladder” or “1 kb plus DNA ladder” were used as size standards. The gels were stained in a plastic tray with 0.005% (w/v) ethidium bromide / TAE for 10 min and then the DNA bands were visualized under UV light. The image was documented with a digital camera.

4.2.5.9. Phenol / chloroform / isoamyl alcohol extraction of DNA with subsequent precipitation by ethanol

The DNA solution obtained after the first digestion with restriction enzyme was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), by using a Vortex mixer. Subsequently the mixture was centrifuged (13,000 rpm, RT, 5 min) in a table top centrifuge. The upper, aqueous, DNA-containing phase was transferred to a new 1.5 ml reaction tube and mixed with 3 volumes of 100 % ethanol. The mixture was incubated at -20°C for 2 h. The pellet obtained by centrifugation (13,000 rpm, 4°C, 10 min), was washed with 80% (v/v) ethanol, air-dried and dissolved in 44 µl ddH_2O, before it was finally subjected to a further digestion with another restriction enzyme.

4.2.5.10. Ligation of PCR products into vector DNA

Based on 25 ng vector DNA, the amount of insert was calculated with the equation:

$Amount_{Insert}$= amount $_{vector}$ X $length_{Insert}$ / $length_{Vector}$.

The dimensions of the amounts of insert and vector were ng and of their lengths were bp.

Vector DNA (25 ng) and the corresponding amount of insert DNA were mixed with 1 µl 10-fold concentrated ligation buffer and 1 µl T_4 ligase (5 U/µl). The reaction mixture was then made up to a final volume of 10 µl with ddH_2O, spinned for 5 s in a microcentrifuge to get the liquid to the bottom of the tube and incubated at 16°C overnight. On the next day, the enzyme was heat-inactivated by incubating the mixture at 65°C for 10 min.

4.2.5.11. Preparation of E.coli cells competent for transformation

Solutions

- TFB I buffer: 30 mM potassium acetate / 100 mM RbCl/ 10 mM $CaCl_2$ / 50 mM $MnCl_2$ / 15% (v/v) glycerol; pH 5.8
- TFB II buffer: 10 mM MOPS / 10 mM RbCl/ 75 mM $CaCl_2$ / 15% (v/v) glycerol; pH 7.0

Experimental procedure

E. coli DH5α cells (Like many cloning strains, DH5 alpha has several features that make it useful for recombinant DNA methods) were kindly provided by Dr. F. Madeo on an agar plate. From this, a suspension culture (5 ml) was prepared in antibiotic-free LB medium overnight. For preparation of transformation competent cells, 500 ml antibiotic-free LB medium was inoculated with 1 ml of the overnight culture and the mixture incubated on a shaking platform at 37°C until the bacteria suspension reached an OD of 0.5 at a wavelength of 595 nm. Then cells were centrifuged down in a Heraeus Varifuge K (3,000 rpm, 4°C, 15 min). The resulting pellet was resuspended in 150 ml TFB I buffer, incubated on ice for 15 min and subsequenly centrifuged at 3,000 rpm, 4°C for 15 min. The resulting pellet was resuspended in 20 ml of TFB II buffer. Finally, 100 µl aliquots of the suspension of DH5α competent cells were frozen in liquid nitrogen and stored at -80°C until further use.

4.2.5.12. Transformation of competent bacterial cells with plasmid DNA

Transformation of NovagenBlue Singles™ competent cells

A frozen suspension of NovagenBlue Singles™competent cells (50 µl) was thawed on ice. The suspension was mixed with 1.5 µl of the ligation solution and incubated on ice for 5 min. After this the cells were heat-shocked at 42°C for 30 s. After incubation on ice for 2 min, the cell suspension was mixed with 250 µl super optimal broth medium (SOC medium, provided with the cells, Novagen) and incubated at 37°C on a shaking platform for 1 h. Cell suspensions in volumes of 50-120 µl were plated on carbenicillin-containing agar plates. Then the plates were incubated at 37°C overnight. On the next day, the plates were checked for the presence of colonies.

Transformation of DH5α competent cells

A suspension of frozen DH5α competent E. coli cells (50 µl) was thawed on ice, mixed with 10 µl of the ligation solution (4.2.5.10) and kept on ice for 10 min. Then a heat-shock of 42°C was applied for 2 min. Subsequently antibiotic-free LB medium (900 µl) was added, and the suspension was incubated on a shaking platform at 37°C for 1 h. The process was continued as described for the transformation of NovagenBlue Singles™ competent cells (see above).

4.2.5.13. Reverse transcription of RNA

Total RNA was isolated from EPCs with the RNeasy RNA isolation kit according to the manufaturer's advice (4.2.5.2). The reaction mixture for reverse transcription of RNA was prepared using 5 mM $MgCl_2$, 10 mM each of dATP, dGTP, dCTP and dTTP, 25 U avian myoblastosis virus reverse transcriptase (AMV RT), 0.6 µg oligo (dT_{15}) primer, 0.6 µg random hexamer primer and 1 µg total RNA in 50 µl of PAN Script NH_4 buffer (Pan Biotech GmbH) and incubated at 42°C for 1 h. The reaction was stopped by heating at 95°C for 5 min. The resulting solution was diluted with an equal amount of H_2O and stored at -80°C. The reaction mixture lacking RT was used as a negative control.

4.2.5.14. Replacement of CMV promoter with wdr16 full length promoter

In order to have an internal standard for the deletion analysis experiment the CMV promoter which drives the SEAP in the pWPXL plasmid was replaced with the wdr16 full length promoter (766 bp). The wdr16 full length promoter with restrictions sites SalI and PacI was amplified from rat genomic DNA using the following primer pair:

5'- CCATTATCGTTTCAGACCCACCTC - 3'
5'- TGTATGTCTGTTGCTATTATGTCTACTATTCTTTC - 3'

For the PCR *PfuUltra™* hot start high-fidelity DNA polymerase was used. Before thermal cycling, the samples were heated to 95°C for 2 min. The cycling parameters were: 95°C, 30 s; 45°C, 30 s; 72°C, 1 min (35 times); final elongation: 72°C, 10 min. The ensuing PCR product was purified with the NucleoSpin® Extract II kit according to the manufacturer's instructions. Thus purified wdr16 promoter PCR product was digested with SalI, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with pacI.

The pWPXL vector was simultaneously digested with restriction nucleases SalI and PacI and the larger digestion product of two bands was purified by preparative agarose gel electrophoresis. The digested pWPXL vector DNA and the purified wdr16 promoter PCR product were then ligated with the aid of T4 ligase, the ligation products were transferred into DH5α competent E.coli cells, and the transformed cells were selected on carbenicillin (50 µg/ml) containing agar plates. Agar streak plates were prepared from colonies that had grown during an overnight incubation. These colonies were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNAs were subjected to digestion with EcoRI and the respective products were analysed on analytical 1% agarose gels. The plasmids showing the expected band pattern were commercially sequenced over their insert region by using sequencing primers.

4.2.5.15. Cloning of the adapter bearing the recognition site for restriction by *Spl*I

In order to clone the full length 766 bp wdr16 promoter in front of the reporter gene FFL, an adapter containing the recognition site of the restriction endonulease SplI was produced by annealing the oligonucleotides

5′ -AATTCCGTACGGGCGCGCCCG- 3′

5′ -GACCGGGCGCGCCCGTACGG- 3′

with each other and then inserting them between the *Rsr*II site and the *Eco*RI site of the pWPXL plasmid. The oligonucleotides, synthesized commercially by Eurofins MWG Company, were suspended at the same molar concentration. Five microliter of each complementary oligo nucleotide was mixed with 40 µl of annealing buffer (10 mM Tris/HCl, pH 7.5; 50 mM NaCl, 1 mM EDTA) in a 1.5 ml centrifuge tube. Then the tube was placed in a standard heating block at 95°C for 4 min and subsequently kept at 70°C for 10 min. Thereafter it was allowed to cool down at RT for 1 h. The annealed oligonucleotides were then digested with RsrII followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with EcoRI (4.2.5.9). The pWPXL vector was digested with RsrII and EcoRI and the larger digestion product out of the two fragments was purified by preparative agarose gel electrophoresis.

The larger digestion product of pWPXL vector DNA and the annealed oligonuleotides (adapter) were then ligated with the aid of T4 ligase (4.2.5.10), the ligation products were transferred into DH5α competent E.coli cells and the transformed cells were selected on carbenicillin-containing agar plates (50 µg /ml). Agar streak plates were prepared from colonies that had grown during an overnight incubation. These plates, in turn, were used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNAs were subjected to digestion with EcoRI and the digestion products analyzed on analytical 1% agarose gels. The plasmids showing the expected band pattern were commercially sequenced over their insert region by using sequencing primers.

4.2.5.16. Cloning of full length wdr16 promoter and of deleted fragments of wdr16 promoter

In order to perform deletion analysis experiments, the full length wdr16 promoter and different deleted fragments of the wdr16 promoter were cloned in front of the FFL reporter gene in the pWPXL plasmid which also contained SEAP under the control of the wdr16 full length promoter. Four different constructs were made, namely CTRL, C1, C4 and C5. The desired wdr16 promoter fragments were amplified by PCR from genomic EPC DNA using the following primers:

CTRL - 5′ -TTCCGTACGAACCTGTTTTATTGACCTC- 3’
5′ -GGCGGACCGTTCAGCTGGTTGATT- 3′

C1 - 5′ -TTCCGTACGGAAAGGAAAATGGTTACA- 3’
5′ -GGCGGACCGTTCAGCTGGTTGATT- 3′

C4 - 5′ -TTCCGTACGTTCCATGCTTTTTTTAAG- 3′
5′ -GGCGGACCGTTCAGCTGGTTGATT- 3′

C5 - 5′ -TTCCGTACGTTTATAGTTTAAAAAAAGTCACAGA- 3′
5′ -GGCGGACCGTTCAGCTGGTTGATT- 3′

Before thermal cycling, the samples were heated to 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 45°C, 30 s; 72°C, 1 min (35 times); final elongation: 72°C, 10 min. The PCR products were then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer’s instructions. The purified wdr16 promoter PCR products were digested with RsrII, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with SplI.

The pWPXL vector was simultaneously digested with RsrII and SplI and the larger digestion product out of the two fragments was purified by preparative agarose gel electrophoresis. The digested pWPXL vector DNA and the desired fragments of wdr16 obtained from PCR were then ligated with the aid of T4 ligase. The ligation products were transferred into DH5α competent E.coli cells and the transformed cells were selected on carbenicillin-containing (50 μg /ml) agar plates. Agar streak plates were prepared from colonies growing

after overnight incubation. These were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNAs from CTRL construct were subjected to digestion with *EcoRI* and the respective products were analysed on analytical 1% agarose gels. The isolated plasmid DNAs from C1, C4 and C5 constructs were subjected to digestion with *SplI* and *RsrII* and the respective products were analysed on analytical 1% agarose gels. The plasmids showing the expected band pattern were commercially sequenced over their insert region by using sequencing primers.

4.2.5.17. Replacement of SEAP with Renilla luciferase

Since SEAP was not a sufficiently sensitive indicator, it was replaced with Renilla luciferase in the pWPXL vector. The Renilla luciferase gene was amplified from the pRL-CMV vector. The following primers with restriction endonuclease sites for MluI and SplI were used to amplify the Renilla reporter gene:

5'- TACGCGTATGACTTCGAAAGTTTATGATCC - 3'
5' - TTCGTACGTTGTTGTTAACTTGTTTATTGCAG - 3'

PfuUltra™ hot start high-fidelity DNA polymerase was used during the PCR. The cycling parameters were: 95°C, 30 s; 55°C, 30 s; 72°C, 4 min (30 cycles); final elongation: 72°C, 10 min. Before thermal cycling, the samples were heated at 95°C for 2 min. The PCR product was then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions. The purified PCR product was digested with MluI, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with SplI.

The pWPXL vector DNA also was digested with MluI and SplI and dephosphorylated with CIAP in order to prevent vector self-ligation in the subsequent ligation reaction. The dephosphorylated vector was purified by preparative agarose gel electrophoresis. The purified pWPXL vector DNA and the purified Renilla PCR product were then ligated with the

aid of T4 ligase. The ligation products were transferred into DH5α competent E.coli cells. The transformed cells were selected on carbenicillin-containing (50 μg /ml) agar plates.

Agar streak plates were prepared from colonies growing after overnight incubation. These were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNAs were subjected to digestion with NotI and the respective products were analysed on analytical 1% agarose gels.

4.2.5.18. Replacement of Renilla luciferase gene with GSL gene

Since Renilla luciferase activity was not detectable in the deletion analysis experiments, it was replaced by GSL. The GSL gene was amplified from the plasmid pCMV-Gluc bought from New England BioLabs. The following primers with the restriction endonuclease sites for MluI and SpI were used to amplify the Gaussia luciferase gene

5' - TACGCGTGCTTAATGCTTGGCTATCG - 3'
5' - TTCGTACCCTTATGCCGGATCCGCATCG - 3'

Before thermal cycling, the samples were heated in the thermocycler at 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 50°C, 30 s; 72°C, 4 min (30 times); final elongation: 72°C, 10 min. The PCR product was then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions.The purified Gaussia PCR product was digested with MluI, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with SplI.

Also the pWPXL vector DNA was digested with MluI and SplI and dephosphorylated with CIAP in order to prevent vector self-ligation in the subsequent ligation reaction. The dephosphorylated vector was purified by preparative agarose gel electrophoresis. The purified pWPXL vector DNA and the purified PCR product were then ligated with the aid of T4 ligase. The ligation products were transferred into DH5α competent E.coli cells. The transformed cells were selected on carbenicillin-containing (50 μg /ml) agar plates. Agar streak plates were prepared from colonies that had grown during an overnight incubation.

They were used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNAs were subjected to digestion with NotI and the respective products were analysed on analytical 1% agarose gels.

4.2.5.19. Cloning of constructs C1A, C1B and C1C (25 bp deletion analysis of the first 100 bp region of the full length wdr16 promoter)

Three constructs, namely C1A, C1B and C1C, were produced for the 25 bp deletion analysis of the first 100 bp region of the full length promoter. The deleted fragments of the full length promoter were cloned upstream of the FFL reporter gene in the pWPXL vector which also had the Gaussia luciferase gene under the control of full length wdr16 promoter. The desired fragments were amplified from the genomic EPC DNA by using the following primers:

C1A: 5'- CGTACGACGCCCCAAAGCGC - 3'
5' - CGGACCGTTCAGCTGGTTGATTCCT - 3'

C1B: 5' - CGTACGGACAGTGGGTTGCAAGAATC - 3'
5' - CGGACCGTTCAGCTGGTTGATTCCT - 3'

C1C: 5' - CGTACGTTGTTAATTTACATGAATTGCTGGAG - 3'
5' - CGGACCGTTCAGCTGGTTGATTCCT - 3'

Before thermal cycling, the samples were heated in the thermocycler at 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 45°C, 30 s; 72°C, 1 min (35 times); final elongation: 72°C, 10 min. The PCR products were then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions. The purified fragments of wdr16 promoter PCR products were digested with RsrRII, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with SplI. The pWPXL vector was simultaneously digested with RsrII and SplI and the larger digestion product was purified by preparative agarose gel electrophoresis. The digested pWPXL vector DNA and the desired fragments of wdr16

promoter were then ligated with the aid of T4 ligase. The ligation products were transferred into DH5α competent E.coli cells and the transformed cells were selected on carbenicillin-containing (50 μg /ml) agar plates. Agar streak plates were prepared from colonies that had grown during an overnight incubation. They were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated plasmid DNAs from C1A, C1B or C1C constructs were subjected to digestion with *Eco*RI and the respective products were analysed on analytical 1% agarose gels. The plasmids showing the expected band pattern were commercially sequenced over their insert region by using sequencing primers.

4.2.5.20. Cloning of C1C-1, C1C-2, C1C-3 and C1C-4 constructs (5 bp deletion analysis of the core promoter region)

Four different constructs namely C1C-1, C1C-2, C1C-3 and C1C-4 were produced for the 5 bp deletion analysis of the core promoter region. The deleted fragments of the full length promoter were cloned upstream of the FFL reporter gene of vector pWPXL which also had the Gaussia luciferase gene under the control of the full length wdr16 promoter. The desired fragments were amplified from the genomic EPC DNA by using the following primers:

C1C-1: 5' - CTGCGTACGATTTACATGAATTGCTGGAG - 3'
5' - TGGCGGACCGTTCAGCTGGTT - 3'

C1C-2: 5' - CTGCGTACGCATGAATTGCTGGAG - 3'
5' - TGGCGGACCGTTCAGCTGGTT - 3'

C1C-3: 5' - CTGCGTACGATTGCTGGAGGAAAGG - 3'
5' - TGGCGGACCGTTCAGCTGGTT - 3'

C1C-4: 5' - CTGCGTACGTGGAGGAAAGGAAAATG - 3'
5' - TGGCGGACCGTTCAGCTGGTT - 3'

Before thermal cycling, the samples were heated in the thermocycler to 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 45°C, 30 s; 72°C, 1 min (35 times); final elongation: 72°C, 10 min. The PCR products were then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions. The purified fragments of the PCR products derived from the wdr16 promoter were digested with *Rsr*II. This procedure was followed by a phenol/chloroform/isoamyl alcohol extraction and subsequent ethanol precipitation, and a further digestion with *Spl*I.

The pWPXL vector was simultaneously digested with *Rsr*II and *Spl*I and the larger digestion product was isolated by preparative agarose gel electrophoresis. The digested pWPXL vector DNA and either the purified wdr16 promoter or the PCR products of the deleted promoter were then ligated with the aid of T4 ligase. The ligation products were transferred into DH5α competent *E.coli* cells. The transformed cells were selected on carbenicillin-containing (50 μg/ml) agar plates that had grown during an overnight incubation. These were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The plasmid DNAs isolated from C1C-1, C1C-2, C1C-3 and C1C-4 constructs were subjected to digestion with *EcoR*I and the respective products were analysed on analytical 1% agarose gels. The plasmids showing the expected band pattern were commercially sequenced over their insert region by using sequencing primers.

4.2.5.21. Cloning of "Turn Around" wdr16 promoter in front of the FFL gene in the CTRL vector

To obtain a plasmid in which the orientation of the full length wdr16 promoter in front of the FFL gene is inverted (for "INV" virus production), the recognition sites for the restriction enzymes *Rsr*II and *Spl*I were swapped on the primers used for cloning the wdr16 promoter into the CTRL viral vector. The following are the primers in which restriction sites were swapped in respect to the CTRL primers (4.2.5.16):

5' - CGGACCGAACCTGTTTTATTGACCTC - 3'

5' - CGTACGTTCAGCTGGTTGATT - 3'

Before thermal cycling, the samples were heated in the thermocycler at 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 45°C, 30 s; 72°C, 1 min (35 times); final elongation: 72°C, 10 min. The PCR products were then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions. The purified PCR product was digested with *Rsr*II, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation, and a further digestion with *Spl*I.

The pWPXL vector was simultaneously digested with *Rsr*II and *Spl*I and the larger digestion product was purified by preparative agarose gel electrophoresis. The digested pWPXL vector DNA and the purified PCR product were then ligated with the aid of T4 ligase, the ligation products were transferred into DH5α competent *E.coli* cells and the transformed cells were selected on carbenicillin containing (50 μg/ml) agar plates. Agar streak plates were prepared from colonies that had grown during an overnight incubation. These were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNA from INV construct were subjected to digestion with *EcoRI* and the respective products were analysed on analytical 1% agarose gel.

4.2.5.22. Replacement of wdr16 promoter in front of the FFL gene with the EF1α promoter

The wdr16 promoter in front of the the FFL gene in the pWPXL vector was replaced with the EF1α promoter. The Ef1α promoter was amplified using the following primers containing the restriction sites for *Rsr*II and *Spl*I:

5' - CGTACGAAGCTTTGCAAAGATGGATA - 3'

5' - CGGACCGTCACGACACCTGA - 3'

Before thermal cycling, the samples were heated in the thermocycler at 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 55°C, 30 s; 72°C, 4 min (30 times); final elongation: 72°C, 10 min. The PCR product was then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions. The purified PCR product

(EF1α promoter) was digested with *Rsr*II, followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation, and a further digestion with *Spl*I.

The pWPXL vector was simultaneously digested with *Rsr*II and *Spl*I to cut out the wd16 full length promoter. The larger digestion product was purified by preparative agarose gel electrophoresis. The digested pWPXL vector DNA and the purified PCR product of the EF1α promoter were then ligated with the aid of T4 ligase. The ligation products were transferred into DH5α competent *E.coli* cells and the transformed cells were selected on carbenicillin-containing (50 μg/ml) agar plates. Agar streak plates were prepared from colonies that had grown during an overnight incubation. These were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNA was subjected to digestion with *SplI* and *RsrII* and the respective products were analysed on analytical 1% agarose gels.

4.2.5.23. Cloning of sox5S and sox5L under the control of the EF1α promoter in vector pWPXL

The sox5S and sox5L genes were cloned into pWPXL, where they were put under the control of the EF1α promoter. The sox5S gene and the sox5L gene followed by the Flag gene were amplified from the sox5 gene using expression plasmids which were kindly provided by Dr. Veronique Lefebvre.The following were the primers used for amplifying the sox5S and sox5L genes with the restriction sites for RsrII and SpeI:

Sox5S: 5' - TGACGGTCCGGATCTTGGTGGCG - 3'
5' - ATGACTAGTTCAGTTGGCTTGTCCCGCAATG - 3'

Sox5L: 5' - TGACGGTCGTATTGCGTTAGCAACGCC - 3'
5' - TGACTAGTTCAGTTGGCTTGTCCCGCAATG - 3'

Before thermal cycling, the samples were heated in the thermocycler at 95°C for 2 min. The PCR cycling parameters were: 95°C, 30 s; 50°C, 30 s; 72°C, 4 min (30 times); final elongation: 72°C, 10 min. The PCR products were then purified with the NucleoSpin® Extract II kit from

Macherey-Nagel according to the manufacturer's instructions. The purified PCR products derived from the Sox5S and Sox5L genes were digested with *Rsr*II. This was followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with *Spe*I. For cutting out the FFL gene the pWPXL vector was simultaneously digested with *Rsr*II and *Spe*I. The larger digestion product was purified by preparative agarose gel electrophoresis. The digested pWPXL vector DNA, the purified PCR products Sox5S or Sox5L genes were then ligated with the aid of T4 ligase. The ligation products were transfected into DH5α competent *E.coli* cells and the transformed cells were selected on carbenicillin-containing (50 μg/ml) agar plates. Agar streak plates were prepared from colonies that had grown during an overnight incubation. These were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated plasmid DNAs containing sox5S or sox5L constructs were subjected to digestion with BamHI and the respective products were analysed on analytical 1% agarose gel.

4.2.5.24. Cloning of full length wdr16 promoter in plasmid pHIS2.1

The wdr16 full length promoter was cloned upstream of the His3 reporter in pHIS2.1. The full length wdr16 promoter was amplified from the genomic EPC DNA with the restriction sites SacI upstream and MluI downstream. The following primers were used to amplify the full length wdr16 promoter:

5' - GAGCTCTTCAACCTGTTTTATTGACCTCAGAAC - 3'

5' - ACGCGTCTCCAGCAATTCATGTAAATTAACAGCGCTC - 3'

Before thermal cycling, the samples were heated in the thermocycler at 95°C for 2 min.The PCR cycling parameters were: 95°C, 30 s; 45°C, 30 s; 72°C, 1 min (35 times); final elongation: 72°C, 10 min. The PCR products were then purified with the NucleoSpin® Extract II kit from Macherey-Nagel according to the manufacturer's instructions. The purified PCR product derived from the wdr16 promoter was digested with *Sac*I. This procedure was followed by a phenol/chloroform/isoamyl alcohol extraction with subsequent ethanol precipitation and a further digestion with *Mlu*I. The pHIS2.1 vector was simultaneously digested with *Sac*I and *Mlu*I and the larger digestion product was purified by preparative agarose gel

electrophoresis. The digested pHIS2.1 vector DNA and the purified PCR product derived from the wdr16 promoter were then ligated with the aid of T4 ligase. The ligation products were transfected into DH5α competent *E.coli* cells and the transformed cells were selected on carbenicillin-containing (50 μg/ml) agar plates. Agar streak plates were prepared from colonies growing after overnight incubation, which were in turn used to start bacterial liquid cultures, from which the recombinant plasmid DNA was isolated with the NucleoSpin® Plasmid kit according to the manufacturer's protocol. The isolated recombinant plasmid DNA was subjected to digestion with *EcoR*I and the respective products were analysed on analytical 1% agarose gels.

4.2.6. Discontinuous SDS-PAGE

SDS-PAGE was carried out as described by Laemmli et al. (1970) with the modifications by Garfin (1990)

4.2.6.1. Solutions

- Acrylamide solution: 29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (Rotiphorese Gel 30)
- SDS: 10% (w/v)
- APS solution: 10% (w/v)
- TEMED
- Running gel buffer: 0.5 M Tris/HCl, pH 8.9
- Stacking gel buffer: 1.5 M Tris/HCl, pH 6.8
- Electrode buffer: 25 mM Tris/HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3
- Five-fold concentrated sample buffer: 0.16 M Tris/HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 0.38 M mercaptoethanol, 0.008 (w/v) bromophenol blue; pH 6.8

4.2.6.2. Preparation of the gels

The gel size was 8 cm x 10 cm x 1 mm. The stacking gels were prepared with 3% acrylamide at pH 6.8, the running gels with 10% acrylamide at pH 8.9. The scheme shown in Table 3 was used for casting the gels.

Table 3. Scheme of the preparation for PAGE of stacking and running gels

Solution	10% running gel	3% stacking gel
Acrylamide solution	3.33 ml	0.50 ml
Running gel buffer	2.5 ml	
Stacking gel buffer		1.25 ml
Water	4.05 ml	3.0 ml
degassing (2 min)		
10% (w/v) SDS	100 µl	50 µl
TEMED	20 µl	5 µl
10% (w/v) APS solution	37.5 µl	200 µl

4.2.6.3. Preparation of the samples

Protein solution was mixed with 5-fold concentrated sample buffer and filled up with ddH_2O to yield a final concentration of 1-fold concentrated sample buffer in a maximal volume of 18 µl. The mixture was then heated to 95°C for 7 min. After collecting the condensate by brief centrifugation, the samples were applied to the gel.

4.2.6.4. Electrophoresis

Electrophoresis was carried out under a constant current of 20 mA at RT. When the bromophenol blue front had reached the end of the running gel, the process was stopped. The gels were either stained with Coomassie Brilliant Blue R 250 or used for Western blotting.

4.2.6.5. Coomassie blue staining of PAGEs

Solutions

- Staining solution: 50% methanol, 40% H_2O, 10% glacial acetic acid, 1% (w/v) Coomassie Brilliant Blue R 250 (in water)
- Destaining solution: Methanol: H_2O: glacial acetic acid (3: 6: 1)

Experimental procedure

Polyacrylamide gels were incubated with staining solution at RT for 10 min. Thereafter, gels were briefly rinsed with water and transferred to destaining solution. When the blue background had disappeared and single protein bands had become visible, the destaining solution was discarded and the gels were washed with ddH_2O. Protein bands were documented by a digital camera

4.2.6.6. Western blot analysis with chemiluminescence detection

Protein Transfer Solutions

- Transfer buffer: 25 mM Tris/HCl, 192 mM glycine; pH 9.0
- Ponceau S solution: 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid

Experimental procedure

Proteins (20 µg) were separated by discontinuous SDS PAGE. The protein bands were then transferred from the gel to a nitrocellulose membrane using a modification of the protocol described by Burnette (1981), as detailed below.

The nitrocellulose membrane was rinsed in transfer buffer. The transfer "sandwich" was packed by piling a plastic lattice, a synthetic fiber mat, a filter paper, the SDS polyacrylamide gel, the nitrocellulose membrane, a filter paper, a synthetic fiber mat and a final plastic lattice. Air bubbles were strictly avoided in the process. The "sandwich" was inserted into an electroblot chamber filled with transfer buffer. Electrophoretic protein transfer was performed with a current of 140 mA at 4°C for 2 h. After transfer, the membrane was removed from the transfer chamber and stained by brief immersion in Ponceau S solution.

Subsequently the membrane was destained with 0.05% (w/v) Tween 20 / PBS, washed with PBS and processed as described in the following section.

Detection of protein bands with the enhanced chemiluminescence (ECL) reagent

Solutions

- Washing buffer: 20 mM Tris/HCl, 150 mM NaCl, 0.02% (w/v) Tween 20; pH 7.4
- Blocking solution: 1-fold concentrated Roti-Block / PBS
- Substrate solution: 1 ml ECL solution I + 1 ml ECL solution II

4.2.7. Yeast one hybrid System

4.2.7.1. Media used for culturing yeast

1 M 3-amino-1,2,4-triazole (3-AT): The solution was prepared in deionized water, and filter-sterilized prior to storage at 4°C.

YPD medium: 10 g yeast extract, 20 g bacto-pepton, dissolved in 900 ml of H_2O and autoclaved, then cooled down to RT before 100 ml of sterile dextrose (20%) was added under sterile conditions.

SD medium: is composed of the following components: Yeast nitrogen base without amino acids (6.7 g), 20 g agar (for plates only), 850 ml of water, and 100 ml of the appropriate sterile 10X dropout solution. The medium was autoclaved and allowed to cool to ~55°C before the addition of 3-AT. Glucose was added to yield a final concentration of 2% (w/v). For 3-AT containing medium, the appropriate amount of 1 M 3-AT stock solution was added and the medium was mixed well.

4.2.7.2. Generation of a cDNA library from total EPC RNA

A cDNA library was generated from total EPC RNA by following the user's manual of the Match maker One-Hybrid Library construction & Screening Kit. The procedure consisted of the following three steps:

1. First-strand cDNA synthesis

2. Amplification of cDNA by LD-PCR
3. Column purification of ds cDNA with a CHROMO SPIN TE-400 column.

Total RNA was extracted from EPC according to the user's manual for the Roche High Pure RNA Isolation Kit.

First-strand cDNA synthesis

Total RNA (2 µl; 1 µg) from EPC was placed in a sterile microcentrifuge tube (50 µl). Then 1 µl of CDS III primer was added and the volume made up to 4 µl by adding 1 µl of deionised water. The tube was incubated at 72°C for 2 min and then allowed to cool on ice for 2 min. Then the following ingredients were added and mixed by tapping

2.0 µl 5X First strand buffer
1.0 µl DTT (20 mM)
1.0 µl dNTP Mix (10 mM)
1.0 µl MMLV reverse transcriptase

The reaction mixture was incubated at 42°C for 10 min before 1 µl of SMART III Oligos was added, the fluids were mixed and the mixture was incubated at 42°C for 1 h. After the incubation, the tubes were placed at 75°C to terminate the first strand synthesis and then allowed to cool to RT. After addition of 1 µl of RNase H (2 units) the reaction mixture was incubated at 37°C for 20 min. The solution obtained was used for the Long Distance PCR (LD-PCR) amplification.

Amplification of cDNA by LD-PCR

For the amplification of cDNA by PCR, the following ingredients were mixed:

- 2 µl first strand cDNA
- 70 µl deionised water
- 10 µl 10X Advantage 2 PCR buffer (Clontech Laboratories)
- 2 µl 50X dNTP Mix
- 2 µl 5' PCR primer

- 2 µl 3’ PCR primer
- 10 µl 10X GC melt solution
- 2 µl 50X Advantage 2 Polymerase Mix

The thermal cycling was carried out using the following settings: 1) 95°C, 30 s; 2) 22 cycles of a) 95°C, 10 s; b) 68°C, 6 min; 3) Final elongation: 68°C, 5 min

The PCR product was analysed by using 7 µl of the product alongside 0.25 µg of a 1 kb DNA size marker on a 1.2% agarose gel.

Purification of ds cDNA with a CHROMO SPIN TE-400 column

One CROMO SPIN TE-400 column was prepared for 93 µl of the cDNA sample. The column was inverted several times to resuspend the gel matrix completely. The “breakaway” was snapped off from the bottom of the column and discarded. The column was placed in a 2 ml collection tube. Then the top cap was removed. The column in the 2 ml collection tube was centrifuged at 700 g for 5 min to purge the equilibration buffer. The buffer in the collection tube was discarded and the column was replaced in another collection tube. The sample (93 µl) was applied to the center of the flat surface of the gel matrix in the column. Centrifugation of the column at 700 g for 5 min then allowed collection of the purified sample.

The purified cDNA was ethanol-precipitated by adding one tenth volume of 3 M sodium acetate buffer (pH 5.3) followed by 2.5 volumes of ice-cold ethanol, placing the mixture at -20°C for 1 h, and then centrifuging (RT, 14,000 rpm) for 20 min. The supernatant was discarded without disturbing the pellet. The centrifuge tube was then again centrifuged briefly at 14,000 rpm to remove the remaining supernatant. The pellet was air dried for 10 min and then resuspended in 20 µl of deionised water. The cDNA was then used for the library construction by *in vivo* recombination in yeast.

4.2.7.3. Transformation of yeast cells by the lithium acetate method

The protocol for lithium-acetate mediated transformation of yeast (Gietz et al., 1995) was adapted to the present purpose by slight changes. Yeast colonies grown on YPD plates (4.2.7.1) were picked and washed in 1 ml of sterile water and harvested by centrifugation (5,000 g, RT; 1 min). Cells were then resuspended in a 1.5 ml tube with 1 ml of 100 mM lithium acetate and incubated at 30°C for 5 min while shaking at 330 rpm. Cells were again collected by centrifugation (5,000 g, RT; 1 min). Then various solutions were added to the cell pellets in the following order: 240 µl 50% (w/v) polyethylene glycol 3350, 55 µl water, 36 µl 1 M lithium acetate, 5 µl plasmid DNA (0.1-10 µg) and 10 µl of 5 mg/ml heat denatured salmon sperm carrier DNA. The pipetted solutions were mixed for 2 min (Vortex) and incubated at 42°C for 30 min while shaking. Cells were sedimented by centrifugation (5,000 g, RT; 1 min), resuspended in 100 µl of water and plated onto agar plates with dropout solutions (4.2.7.1) lacking histidine, leucine and tryptophan. Plates were incubated at 30°C for 3 to 7 d to recover transformants. New colonies were streaked onto fresh agar plates and propagated again before inoculation of liquid cultures.

4.2.7.4. DNA extraction from yeast cells

DNA extraction buffer

The numbers in parentheses represent the final concentrations.

- 2 ml 10% Triton-X-100 (2%)
- 1 ml 10% SDS (1%)
- 200 µl 5M NaCl (0.1 M)
- 20 µl 0.5 M EDTA (1 mM)
- 100 µl 1 M Tris/HCl buffer pH 8.0 (0.01 M)
- Sterile water to 10 ml

Yeast colonies were picked up and incubated at 30°C overnight in appropriate medium (2 ml) while shaking at 140 rpm. Cells from 1.5 ml of culture were collected by centrifugation and resuspended in 200 µl of DNA extraction buffer in a 1.5 ml tube. A mixture of phenol-chloroform-isoamylalcohol (25:24:1; 200 µl) was added and the pH value was adjusted to

8.5 with 50 mM Tris/HCl. Thereafter 300 mg of acid-washed glass beads (0.75-1.00 mm in diameter) were added. Cells were broken by treatment with a Vortex mixer for 2 min and centrifuged (16,000 g, RT; 5 min). The upper aqueous layer containing extracted DNA was transferred into a 1.5 ml tube and DNA was precipitated by adding 500 µl of ice-cold ethanol (99%) followed by incubation at -20°C for 1 h. Final sedimentation of DNA was achieved by centrifugation (15,000 g, 2°C; 15 min). The DNA pellet was washed with 300 µl of 70% ethanol, air-dried for 5 min and dissolved in 40 µl of water. The solution of isolated DNA was stored at -20°C.

4.2.8. Electrophoretic mobility shift assay (EMSA)

4.2.8.1. Preparation of nuclear extract from SOX5 overexpressing HEK293T cells

HEK293T cells overexpressing Sox5S or Sox5L were grown in plates 150 mm in diameter to approximately 80% confluency. After removal of the growth medium the cells were washed with PBS containing 1mM EDTA. The cells were collected by centrifugation and resuspended in buffer containing 10 mM Tris/HCl (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM PMSF. Then Nonidet P-40 was added to 0.2% (v/v) and the suspension was mixed vigorously for 30 s and then centrifuged for 30 s at 12,000 rpm. The supernatant was discarded, and the pellet was resuspended in a buffer containing 20 mM Tris/HCl (pH 7.9), 400 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA and 1 mM PMSF. The mixture was vigorously shaken in a 2 ml tube at 4°C for 1 h before it was centrifuged (4°C, 12,000 rpm, 15 min). The supernatant was tranferred to a 1.5 ml tube and the pellet was discarded. The concentration of sox5S and sox5L protein was determined by Bradford assay and 10 µl aliquots of the extracts were stored at -70°C.

4.2.8.2. Labeling of the oligonucleotide probes

The core promoter region wdr16CPR (25 bp) of the full length wdr16 promoter and the consensus binding sites of the sox5S and sox5L genes were synthesized by Invitrogen. The corresponding sequences are:

wdr16CPR: Sense – 5’ - TGTTAATTTACATGAATTGCTGGAG - 3’

Antisense – 5’- CTCCAGCAATTCATGTAAATTAACA - 3’

Consensus binding sites for Sox5S and Sox5L:

Sox5S: Sense – 5’- **AACAAT**GGATCT**AACAAT**GGATCT**AACAAT** - 3’

Antisense – 5’ - **ATTGTT**AGATCC**ATTGTT**AGATCC**ATTGTT** - 3’

(The consensus binding sites of the sox5S gene are presented in boldface)

Sox5L: Sense – 5’- **CATTGAT**GGATCT**CATTGATCATTGAT** - 3’

Antisense – 5’- **ATCAATGATCAATG**AGATCC**ATCAATG** - 3’

(The consensus binding sites of the Sox5L gene are presented in boldface)

First, both the sense and antisense oligonucleotides were labeled separately by using the following protocol: In a 1.5 ml reaction tube 0.5 µl of oligonucleotide (200 ng/µl), 2 µl of the 10X polynucleotide kinase reaction buffer, 0.5 µl of T4 polynucleotide kinase, 1.5 µl of [γ-^{32}P]ATP (at a concentration of 10 mCi/ml = 370 MBq/ml with a specific radioactivity of 3,000 Ci/mmol, corresponding to an ATP concentration of 3.3 µM) were made up to 20 µl with ddH_2O. The reaction mixture for the labeling procedure was incubated at 37°C for 1 h. Thereafter the labeled oligonucleotides were purified using a microspin G-50 column (Amersham). For annealing, the purified sense and antisense oligonucleotides (both 100 µl) were combined and incubated at RT for 10 min.

4.2.8.3. Binding reaction for protein/DNA binding

The binding reaction was carried out on ice except for the final incubation at RT. The total volume of the reaction mixture was 15 µl. For the binding reaction, the components were added in the following order:

1. 5 µl of ddH_2O
2. 1 µl of poly-dIdC (1 µg/µl in TE)
3. 2 µl of binding buffer (5X; 50 mM Tris/ HCl (pH 8.0), 750 mM KCl, 2.5 mM EDTA, 0.5 % (w/v) Triton-X 100, 62.5 % (v/v) glycerol, 1 mM DTT)
4. 6 µl of nuclear extract (5 µg protein; see 4.2.8.1)
5. 1 µl of labeled probe (1:15 dilution)

After addition of all components, the 1.5 ml tube containing the reaction mixture was spun briefly in a microfuge to bring all fluid down to the bottom of the tube. The reaction mixture was incubated at RT for 30 min.

For the competition test, 1 µl of the "cold" competition probe was added to the mixture for the binding reaction before adding the labeled probe.

4.2.8.4. PAGE

Non-denaturating polyacrylamide gels (5%) were prepared for running the EMSA samples. The dimension of the gels was: 20 cm x 17 cm, 1.5 mm spacer, comb with 15 wells. For 50 ml of 5% PA gel were required: 8.3 ml of 30% PA solution (29:1), 2.5 ml of 10X TBE, 39.2 ml of ddH_2O, 50 µl TEMED and 0.5 ml APS.

The EMSA samples (15 µl) were loaded into the wells of the gel which was allowed to run for 2.5 h in 0.5X TBE at 1 mA/cm (at the start the voltage was about 120 V, during the course of electrophoresis the voltage was increased to 200 V). After PAGE, the gel was transferred to a double layer of Whatman filter paper and was dried on the gel dryer (75°C, 1 h). The dried gel was then exposed to an autoradiography film.

4.2.9. Luciferase assays

EPC were treated with the appropriate lentiviral vector on DIV 1 and cultured until the time of the assay. The medium was removed and tested for GSL activity via the Gaussia Luciferase Assay Kit from NEB. At the same time the cells were lysed with the buffer included in the Bright-Glo™ Kit which was subsequently used for assaying FFL activity. Both assay procedures were performed according to the respective manufacturer's instructions.

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6. Summary

1. The goal of the thesis was to characterize the promoter of the gene for Wdr16, a protein specific for kinocilia-bearing cells, by finding the transcription factor binding site and the corresponding transcription factor which regulates the promoter.

2. Ependymal primary cultures proved resistant to transfection with naked plasmid DNA.

3. Lentiviral vector mediated gene transfer in ependymal primary cultures significantly increased the proportion of transfected cells in respect to using other vectors.

4. Ependymal primary culture turned out as an efficient model system for studying the particular effects of transgenes in kinocilia-bearing cells by placing the transgenes under the control of a kinocilia-specific promoter.

5. Ependymal primary cultures proved to be a convenient and well-suited system for characterizing mammalian promoters that are only active in kinocilia-bearing cells.

6. The genomic sequence interspersed between the first exons of the rat stx8 and wdr16 genes was found to act, in the direction of wdr16 transcription, as a promoter specifically activated in kinocilia-bearing cells at the time of kinocilia formation.

7. Due to the proximity of the adjacent stx8 gene, all major promoter elements were expected to be contained within the 766 bp of sequence employed as the promoter region in this work

8. Due to higher signal intensity Gaussia luciferase served as a more reliable reporter for the expression of the wdr16 gene than other luciferases, displaying enhanced stability with respect to challenging experimental conditions.

9. Initial deletion analysis of the promoter showed that in kinocilia-bearing cells the most critical elements for wdr16 expression appear to reside in the 100 base pairs long sequence deleted in the positions -766 to -667 of the full length promoter

10. Bioinformatics analysis of the wdr16 promoter region covered by the probe vectors CTRL, C1, C4 and C5 revealed putative binding sites for eight different transcription factors, including four of the forkhead group.

11. The 766 base pairs of the full length promoter were narrowed down to 10 base pairs (sequence positions -676 to -667) by deletion analysis. The deletion of these 10 base pairs reduced the promoter strength by about 65 %.

12. The characterization of the first 100 base pairs region of the full-length promoter by deletion analysis showed that the last 25 base pairs play a crucial role in the activity of the promoter. A bioinformatics analysis of this sequence revealed Sox5 as the only transcription factor known to be effective at this site.

13. These results of the deletion analysis that Sox5 positively regulates the promoter of the wdr16 gene were confirmed in an electrophoretic mobility shift assay that showed the binding of this transcription factor to its cognate site.