Studies on the function of the transcription factor Sp1 in embryonic stem cells and murine development

Onderzoek naar de functies van de transcriptiefactor Sp1 in embryonale stamcellen en in de ontwikkeling van de muis

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Sokrates, der alte Greis,
Sagte oft in tiefen Sorgen:
"Ach, wie viel ist doch verborgen,
was man immer noch nicht weiss."

Sokrates, the old man,
Often said in deep sorrows:
'Oh, how much is hidden yet,
that we still do not know.'

Aus "Abenteuer eines Junggesellen" von Wilhelm Busch

Meinen Eltern

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Abbreviations

A	Adenine
ATP	adenosine triphosphate
bp	base pair(s)
BRE	TFIIB recognition element
Btd	Buttonhead
cDNA	complementary deoxyribonucleic acid
C	Cytosine
C-terminal	Carboxy-terminal
Cre recombinase	causes recombination
DNA	deoxyribonucleic acid
DPE	downstream promoter element
DSB	double strand break
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
G	Guanine
GTF	general transcription factor
Gy	gray, unit of absorbed radiation dose, 1Gy = 1 joule/kilogram
HAT	histone acetyl transferase
HDAC	histone deacetylase
Inr	Initiator
IVS	intervening sequence, intron
kb	kilo base
lacZ	β-galactosidase
LBD	ligand binding domain
LIF	leukemia inhibitory factor
loxP, lox	locus of X-over (crossing over) of P1
ml	millilitre
mRNA	messenger ribonucleic acid
N-terminal	Amino-terminal
4-OH-T	4-hydroxy tamoxifen
pA	polyadenylation signal
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
PIC	pre-initiation complex
RNA	ribonucleic acid
rpm	rotations per minute
Sp/XKLF	Specificity protein/X Krüppel like factor
T	Thymidine
TAF	TBP associated factor
TBP	TATA box binding protein
UTR	untranslated region
µl	microlitre (10^{-6} litre)

Scope of this thesis

Within this thesis I summarize the results of experiments investigating the *in vivo* functions of the transcription factor Sp1. Since the discovery of Sp1 in the early 1980s, widespread research has been performed and has resulted in several thousand published papers providing some understanding of Sp1 transcriptional regulation. Despite this avalanche of data, the physiological role of Sp1 remains largely elusive. Here I describe experiments aimed at putting a few pieces of the complex Sp1 regulatory function puzzle into place.

In Chapter 1 an introduction on the interface between transcription (the first process to transfer the genetic information of a cell into its effector molecules) and chromatin (the *in vivo* target of the transcriptional machinery). Transcription factors bind DNA at their specific binding sites and are essential to regulate and coordinate the transcriptional machinery with the transcriptional needs of the cell. In this context, the Sp family of transcription factors comprised of 8 members is introduced and the known *in vitro* and *in vivo* functions of these molecules are summarized.
Chapter 2 describes the outcome of our search for target genes of Sp1 by use of the DNA chip technology and Sp1 knock out ES cells.
Chapter 3 describes the new finding that Sp1 knock out ES cells are hypersensitive to ionising radiation, and describes our attempts to discover the molecular mechanism underlying this hypersensitivity.
In Chapter 4 the generation of conditional Sp1 knockout alleles (by use of the Cre-lox system) is described. As the Sp1 knockout results in embryonic lethality, conditional knockout mice are useful to gain insights into Sp1 functions at later stages (fetal and adult) and/or in specific tissues.
Chapter 5 describes the increase of ocular anomalies in Sp1 heterozygous mice in a specific inbred mouse strain.
A General Discussion of the results described in this thesis is given in Chapter 6, together with an outlook on possible future experiments.

Chapter 1

Introduction:

Transcriptional regulation and Sp1-related transcription factors

Transcription and chromatin

The characteristics of a given cell are determined by its protein composition and this protein composition is, in general, determined by the 'turning on/off' of genes. The vertebrate body consists of hundreds of different cell types that all contain the same genes (exceptions are B- and T-cells as those differ in their genetic information due to DNA rearrangements). Hence, cell diversity is brought about by the highly regulated expression of a subset of the total number of genes available in each cell, with each cell lineage being characterized by the expression of specific gene subsets.

The genetic information of DNA is transferred to RNA (via a process called transcription) and from RNA to protein (via a process called translation). In eukaryotes, three RNA polymerases (RNAPI, II, and III) function to transcribe different gene classes (Roeder and Rutter, 1969). RNAPI effects the synthesis of ribosomal RNA (rRNA), while RNAPII transcribes protein-encoding messenger RNAs (mRNA). RNAPIII effects the synthesis of transfer RNAs (tRNA) and other small RNAs.

Transcription of DNA into messenger RNA by RNAPII is one of the most highly regulated processes in the cell. This tight regulation allows the direction of spatial and temporal programs of gene expression, necessary for growth and differentiation of cell lineages during development, as well as their maintenance and reaction to physiological and environmental cues in the adult.

General RNAPII transcription

Transcriptional regulation depends on a complex molecular machine (Figure 1) consisting of an astonishing number of proteins (Lemon and Tjian, 2000). Genes are switched on and off by the interplay of numerous protein factors that interact with each other and with regulatory DNA sequences linked to the specific target genes.

Eukaryotic RNAPII, the protein complex that catalyses DNA-dependent synthesis of mRNA, consists of twelve remarkably conserved subunits (Woychik, 1998). Recent high-resolution data supply insights into the molecular architecture of this machinery (Cramer et al., 2001; Gnatt et al., 2001). Unlike their prokaryotic counterpart, eukaryotic RNAPs are unable to recognize the start region of a gene (promoter) by themselves and therefore they rely on additional factors, so-called general transcription factors (GTFs; e.g. TFIID, TFIIB, TFIIF, TFIIE, TFIIH, TFIIA) for accurate transcriptional initiation (Orphanides et al., 1996; Roeder, 1996).

The assembly of the transcription pre-initiation complex (PIC) consisting of the template DNA, RNAPII and the GTFs, follows a distinct order:

The first GTF to bind DNA is TFIID, a multi-protein complex consisting of the TATA box-binding protein (TBP) and about ten TBP-associated factors (TAFs). The TATA box is an AT-rich sequence located 25-30 bp upstream of the transcriptional start site. TAFs have initially been identified as co-activators (see page 13) that are dispensable for basal transcription but act as molecular adapters between transcription factors and the general transcription initiation machinery (Dynlacht et al., 1991; Pugh and Tjian, 1990; Tanese et al., 1991). The molecular architecture of human TFIID suggests that it acts as a molecular clamp to bind DNA (Andel et al., 1999; Brand et al., 1999). The composition of the protein complex seems to be variable. Not only have TBP-like factors been identified, that bind to DNA sequences different from classical TATA boxes (Dantonel et al., 1999), but it has also become apparent that some TAFs function in a tissue-specific manner (Dikstein et al., 1996; Freiman et al., 2001). TAFs have also been shown to bind the initiator (Inr) sequence, a conserved motif in many promoters that overlaps with the transcriptional start site (Smale and Baltimore, 1989). Binding Inr as well as the DPE (downstream promoter element) (Verrijzer et al., 1995; Verrijzer et al., 1994) enables the TAFs to tether TFIID to the DNA in TATA-less promoters (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997).

TFIIA has been reported to promote the dissociation of TBP- as well as TFIID-dimers, which form via the DNA-binding domain of TBP. This dissociation allows TFIID to load onto promoter DNA (Coleman et al., 1999). TFIIA has been involved in TBP recruitment and stabilization of the TBP-TATA complex. Nevertheless, TFIIA depletion experiments have shown a varying degree of requirement in the activation of different promoters (Liu et al., 1999).

TFIIB enters the PIC subsequently as a prerequisite for the entry of RNAPII. Human TFIIB binds to the TFIIB-recognition element (BRE) which is found in some promoters directly upstream of the TATA box (Lagrange et al., 1998). Defects in human TFIIB have been shown to alter start site selection (Hawkes and Roberts, 1999).

Subsequently the PIC is entered by hypophosphorylated RNAPII, together with TFIIF. As the template DNA has not been unwound so far by the PIC, this state is conferred to as 'closed complex'.

The following addition of TFIIE and TFIIH stimulates the formation of an activated open complex with ATP-dependent isomerization of the complex and 'promoter melting', that is the physical separation of the DNA strands with the formation of the so-called bubble-region. TFIIE forms promoter contacts in and downstream of this bubble region. TFIIH, consisting of nine subunits, is the only GTF with defined enzymatic activities. It includes two ATP-dependent DNA helicases with opposite polarity (XPB and XPD) and a cyclin-dependent protein kinase (cdk7-cyclinH). Structural studies of human TFIIH revealed a ring-like structure with a central hole, big enough to harbour a double-stranded DNA-molecule

(Schultz et al., 2000). The core-TFIIH complex (missing the protein kinase) also plays a major role in nucleotide excision repair (NER) (Wang et al., 1994),

After promoter melting, the first phosphodiester bond of the transcript is formed and the interaction between the RNAPII and the promoter is disrupted ('promoter clearance'). This transition from the initiation mode to the elongation mode is accompanied by massive phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNAPII. Different cyclin-regulated CTD kinases have been identified (e.g. (Murray et al., 2001)). A CTD phosphatase (Chambers and Dahmus, 1994) recycles RNAPII after transcriptional termination to the hypophosphorylated form. CTD phosphorylation also affects co-transcriptional mRNA processes like cap addition to the 5'-end of the transcript, splicing and polyA-addition to the 3'-end of the transcript.

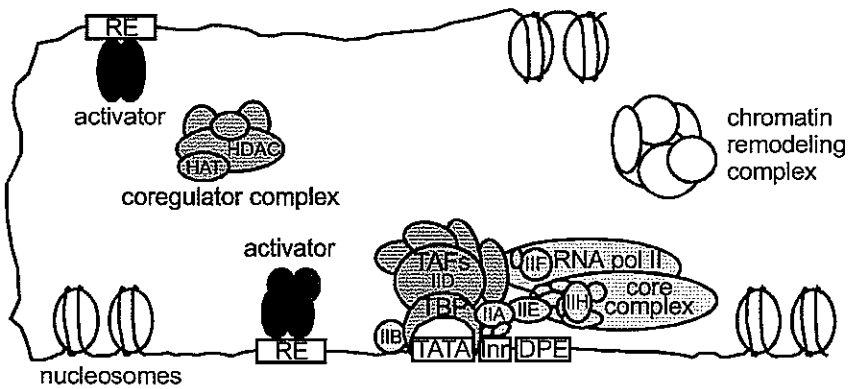

Fig. 1: Key players of transcription.
Depicted is a typical promoter with TATA box, initiator sequence (Inr), downstream promoter element (DPE) and regulatory response elements (RE) for sequence-specific transcriptional activators (black).
The multi-protein complex of the basal transcription machinery (light grey) cooperates with co-regulatory complexes (dark grey) like TFIID and complexes with histone-modifying activities like histone acetyltransferases (HAT) or histone deacetylases (HDACs). Chromatin remodelling complexes and nucleosomes as their chromatin targets are depicted in white. For details see main text. Adapted from (Freiman, 2003).

Chromatin and transcriptional regulation

Most of what is known about the molecular mechanisms involved in general transcription originates from *in vitro* experiments performed with naked DNA templates. However, eukaryotic DNA in its physiologic context within the nucleus of the cell is packaged into a highly organized nucleoprotein structure called chromatin.

The basic unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped almost twice around a protein octamer core consisting of two copies each of the four

positively charged histone proteins H2A, H2B, H3 and H4 (Luger et al., 1997; Thomas and Kornberg, 1975). This structure can be further compacted with the aid of histone H1 and other non-histone proteins to higher-order structures that so far are only poorly characterized. This compaction culminates during mitosis of the cell cycle, when chromosomal DNA becomes highly condensated to form the characteristic metaphase chromosomes.

Increasing data further strengthen the pivotal role of chromatin in the regulation of gene transcription. Along with certain chromatin modifications that are described in the sections 'Co-regulators with histone-modifying activities' and 'DNA methylation', chromatin can acquire different structures (euchromatic or heterochromatic, see Table 1), being more or less accessible to the transcriptional machinery and thereby resulting in transcriptional activation or repression.

Transcription factors

Transcription activators bind in a sequence-specific manner to *cis*-regulatory sites in the promoter and enhancer region of genes in order to activate or repress transcription. These factors interact directly or indirectly with the basal transcription machinery (reviewed in (Mitchell and Tjian, 1989; Ptashne, 1988)). By doing so, they integrate physiological and environmental signals and enable the cell to react to these cues through differential gene expression. As regulatory regions of most genes contain binding sites for multiple transcription factors, a complex regulatory network of protein factors functions to fine-tune gene expression in response to multiple signalling pathways (Lefstin and Yamamoto, 1998).

Transcription factors show a modular structure of a DNA-binding domain and one or more activation domains, sometimes in combination with inhibitory domains. Additional domains involved in the binding of small ligands (e.g. hormone-binding domains like in the family of steroid hormone receptors) or the interaction with other proteins can modulate their activity. Transcription factors are usually classified due to their DNA binding domain. Examples are the Cys2-His2 zinc finger domain of the Sp/XKLF family described in more detail below, or the basic DNA binding domain of the C/EBP-family (Latchman, 1997).

As transcription factors have to deal with usually repressive chromatin structures in order to activate transcription, they can also be subdivided in those that are able to bind to their recognition sequence even in a repressive chromatin context and those that fail to do so. Furthermore, the precise location of the binding site on the nucleosome will influence its accessability (Urnov and Wolffe, 2001). Studies on the induction of the MMTV gene showed the relevance of this different capability of transcription factors (Di Croce et al., 1999; Fletcher et al., 2000). While the glucocorticoid receptor is able to bind to at least some of its cognate sequences within the MMTV promoter when packaged in nucleosomes, binding of

NF1 (nuclear factor 1) to its recognition sequences depends on the activity of chromatin-modifying co-activators (see page 14) that increase the DNA accessability for NF1. These co-activators are recruited to the DNA by the already promoter bound glucocorticoid receptors.

Transcriptional co-activators and co-repressors

The existence of transcriptional co-activators was postulated following the results obtained from Sp1 transcriptional transactivation experiments with purified or semi-purified TFIID fractions. It was found that the Sp1 transcription factor failed to activate transcription in the presence of purified cloned TFIID, whereas it did so in the presence of semi-purified TFIID fractions from *Drosophila* extracts (Pugh and Tjian, 1990). This led to the identification of many additional co-regulators (activators and repressors).

Co-regulators can be recruited to promoters by DNA-bound sequence-specific transcription factors and are required for regulation of gene expression (reviewed in (Hampsey and Reinberg, 1999; Lemon and Tjian, 2000; Naar et al., 2001)). Co-regulators have been divided into different classes according to their properties. Co-regulators can either mediate the interaction between transcription factors and the core transcriptional machinery or (and sometimes additionally) possess chromatin-modifying activities, or be a subunits of a protein complex with these activities.

Co-regulators as protein interfaces

One class of co-regulators encompasses components of the core transcriptional machinery, like for instance the TAFs of the TFIID complex (e.g. (Chen et al., 1994; Hoey et al., 1993)). Another class of co-regulators associates with sequence-specific activators or repressors, thereby modulating their interaction with the core machinery (e.g. (Luo and Roeder, 1995; Strubin et al., 1995).

The third class of co-regulators consists of modular multi-subunit complexes called 'Mediator' that serves as an interface between sequence-specific regulatory proteins and the basal RNAPII machinery. Mediator has first been identified in yeast (Kim et al., 1994; Thompson et al., 1993). Mediator exists as a dynamic set of complexes, which changes in response to specific regulators. These complexes are different in their composition, but usually share some of the subunits. Recently, distantly related human Mediator complexes have bee identified, containing orthologs of several yeast Mediator subunits. Examples are the complexes CRSP (Ryu et al., 1999) and ARC/DRIP/TRAP (Ito et al., 1999; Naar et al., 1999; Rachez et al., 1999). CRSP and ARC are described in more detail in the paragraph 'Sp1 co-activators'.

Co-regulators with chromatin-remodelling activity

Chromatin remodelling complexes use ATP hydrolysis to facilitate access of DNA binding proteins to DNA by repositioning nucleosomes at the promoter or by inducing conformational changes in nucleosomes. Based on the identity of their catalytic ATPase subunit they can be divided into three classes (Boyer et al., 2000), the SWI2/SNF2 family, the ISWI family and the Mi-2 family (reviewed in (Narlikar et al., 2002)).

Co-regulators with histone-modifying activities

Complexes with histone-modifying activities catalyse covalent post-translational modifications of histones but also transcription factors. They can be subdivided further into histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone kinases (Narlikar et al., 2002). While ubiquitination and ADP-ribosylation are also important modifications, they are not considered in the further text.

Targets for these covalent histone modifications are the N-terminal histone tails that protrude from the nucleosome. Various combinations of these modifications have been reported which led to the proposal of a 'histone code' (reviewed in (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Zhang and Reinberg, 2001)). This code of covalent modifications is supposed to be read by proteins that use these as instructions to modulate transitions between different chromatin states.

Acetylation and methylation of histones have long ago been proposed to be involved in gene regulation (Allfrey, 1966). The hyperacetylation of mainly H3 and H4 N-terminal lysine residues by HATs are correlated with transcriptional competence of the chromatin. Conversely, the recruitment of HDACs by transcriptional repressors and subsequent deacetylation of the histone tails is required for transcriptional repression. *In vivo* HATs as well as HDACs function within different multi-protein complexes, providing different interaction sites for different transcriptional regulators.

The NuRD-complex is very interesting, as it not only contains two HDAC subunits, but also the ATP-dependent chromatin remodelling factor Mi-2 (Tong et al., 1998; Wade et al., 1998; Zhang et al., 1998). It has been shown that ATP increases the deacetylase activity of NuRD (Tong et al., 1998) and thus, there is a cooperation of the remodelling activity and the histone modification activity in this complex. The transcriptional repressor protein Ikaros has been shown to recruit NuRD to heterochromatic regions (see Table 1) during T cell activation (Kim et al., 1999). Arginine methylation of histone tails by HMTs so far only has been correlated with transcriptional activation (for review see (Zhang and Reinberg, 2001)). The effect of methylation on lysine residues seems to depend on the position of the lysine within the histone tail. The methylation of lysine 4 on histone H3 by the human HMT Set9 facilitates

transcription (Nishioka et al., 2002). Methylation of lysine 9 of histone H3 by SUV39 however is associated with transcriptional repression (Rea et al., 2000). Recently, it has been shown that the transcriptional repressor HP1 (heterochromatic protein 1) is able to bind to histone H3 when the latter is methylated by SUV39 at lysine 9 (Bannister et al., 2001; Lachner et al., 2001).

Chromatin and epigenetics

The combinatorial modifications at the N-terminal histone tails (described above as 'histone code') are part of an epigenetic marker system that influences transcription and silencing of genes during development, higher-order structures of chromatin, imprinting of genes, X-chromosome dosage compensation and genetic stability. Conversely, deregulation of this marker system has shown its involvement in developmental defects and cancer.

Epigenetic marks increase the information content of chromatin without changing the DNA sequence itself. Nevertheless, they can be stably inherited through generations of cells. Methylation of DNA plays, beside the histone-code, a key role in this system.

DNA methylation

In vertebrates, the methylation state of the cytosine base in the CpG dinucleotide plays an important role in defining transcriptionally active or inactive chromatin regions. DNA methylation is absent in yeast and *Caenorhabditis elegans*. *Drosophila* shows minute levels of DNA methylation (Gowher et al., 2000; Lyko et al., 2000). The pattern of methylated and unmethylated sites is precisely maintained over many cell cycles. However, these methylation patterns change during development and disease. DNA methylation has generally been associated with a transcriptionally repressive, compact chromatin structure. It has been suggested that DNA methylation is a cellular means to suppress transcription from possible deleterious sequences in the genome like proviruses and transposons and to reduce the transcriptional background noise from illegitimate promoters (Bird, 1992).

CpG islands

As methylated cytosines are easily converted to thymidines via deamination, the use of methylated cytosines in the regulation of chromatin structure has lead to an under-representation of the CpG dinucleotide in the genomes of vertebrates caused by a mutagenic pressure on methylated CpGs.

About 80% of these 'left over' CpG dinucleotides are methylated (Gruenbaum et al., 1981). However, small regions of DNA known as 'CpG islands' not only show an almost expected CpG content, but CpGs within these islands are also usually unmethylated not only in the germline but also in most somatic tissues.

CpG islands are often found in association with genes, especially within promoter regions and first exons, but also in more 3'-regions (Larsen et al., 1992). Housekeeping genes are usually associated with CpG islands, but they can also be found in tissue-specific genes (e.g. human α-globin genes (Bird et al., 1987), MyoD1 (Jones et al., 1990), Thy-1 (Antequera et al., 1990).

Aberrant methylation of CpG islands in the promoter of genes often is involved in the silencing of their expression (see Figure 2).

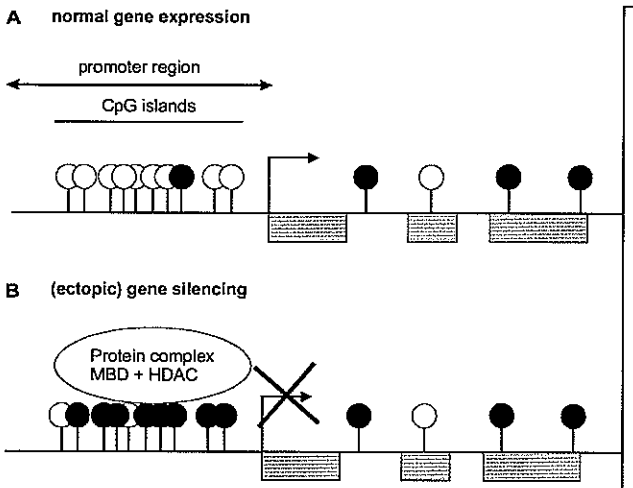

Fig. 2: Influence of CpG island methylation on transcription.
A. The actively transcribed gene shows an unmethylated CpG island in its promoter region.
B. Aberrant methylation of the CpG island leads to recruitment of methyl-CpG binding proteins (MBD) which can interact with histone deacetylases. Binding of these transcriptional co-repressors results in (ectopic) gene silencing. See main text for details. Exons are depicted as grey rectangles, unmethylated CpGs as open, methylated CpGs as filled circles, respectively. Adapted from (Bird, 1992) and (Leonhardt, 2000).

CpG islands normally exist in an 'unmethylated default state' that usually cannot be correlated per se with transcriptional gene activation. The CpG island in the muscle determination gene MyoD1 is unmethylated in expressing as well as in non-expressing tissues like the brain (Jones et al., 1990).

Due to their inherent CG-rich sequence, CpG islands usually encompass binding sites for the transcription factor Sp1 (GGGGCGGGG) (for more information the reader is referred to the paragraph 'The zinc finger connection: A family portrait). Sp1 binding sites have been shown to be essential for the maintenance of the methylation-free status of the CpG island in the housekeeping gene APRT (Brandeis et al., 1994; Macleod et al., 1994).

DNA methyltransferases (DNMT)

The post-replicative addition of methyl-groups to the 5-position of cytosines is catalysed by three different DNA cytosine methyltransferases (DNMT). For all three active DNMTs transcriptional repression capabilities have been described that are independent of catalysing DNA methylation. This repressive capacity is instead due to interactions with other

co-repressors like HDACs (Bachman et al., 2001; Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000).

DNMT1, the first cloned eukaryotic DNA methyltransferase (Bestor et al., 1988) has been shown to have a preference for hemi-methylated DNA and has therefore been assigned a function in the maintenance of methylation patterns after DNA replication. The finding of its association with PCNA (proliferating cell nuclear antigen) and co-localization with DNA replication foci in early S-phase (Chuang et al., 1997) is consistent with the ascribed role in methylation maintenance.

ES cells with homozygous Dnmt1 mutation grow normally although having severely demethylated genomes (Li et al., 1992). However, upon induced differentiation they undergo cell-autonomous apoptosis. Homozygous mutant embryos are delayed in development and die *in utero* around mid-gestation.

As ES cells lacking DNMT1 still show residual DNA methylation (Li et al., 1992) and are capable of methylating introduced retroviral DNA *de novo* (Lei et al., 1996; Okano et al., 1999), the existence of other DNMTs was proposed. Two other active DNMTs (DNMT3a and DNMT3b) have been cloned based on sequence homologies (Okano et al., 1998). DNMT3a knockout mice develop to term, but most of them become runted and die around four weeks after birth. However, the absence of DNMT3b results in embryonic lethality. Embryos develop normally until about embryonic day 9.5, but die soon thereafter, showing variable developmental defects. Compound homozygous knockout embryos showed an even more severe phenotype resulting in developmental arrest shortly after gastrulation (Okano et al., 1999) and suggesting some overlapping functions for DNMT3a and DNMT3b. However, since the single knockout mice display different developmental defects, different functional properties of these two methyl-transferases are implied. Indeed, it appears that DNMT3a and DNMT3b differ in their target sequences, as only DNMT3b is essential for the methylation of minor satellite repeats, repetitive sequences in the centromeric regions of chromosomes (Okano et al., 1999).

This finding is in agreement with the identification of mutations in DNMT3b as causal for ICF (standing for immunodeficiency, centromere instability and facial anomalies). Cells from patients affected by this syndrome show extensive loss of methylation from pericentromeric regions (Xu et al., 1999) of chromosomes 1, 9 and 16. These regions are known to contain classical satellite regions that are normally heavily methylated (Lubit et al., 1976). Undermethylation of these sequences has been correlated with pronounced chromatin decondensation leading to the found elongation of the corresponding regions. Although this centromeric instability can be found in all tissues of the patients, only phytohaemagglutinin-stimulated T cells showed abundant chromosome abnormalities which involve the described

decondensated regions (Xu et al., 1999). Nevertheless, lymphoblastoid cell lines from ICF patients are highly sensitive to ionising irradiation as genotoxic agent (Narayan et al., 2000).

Methyl-CpG-binding proteins

A family of methyl-CpG-binding proteins is known in vertebrates (MeCP2, MBD1-4) that is defined by one structural feature (Nan et al., 1993), the methyl-CpG-binding domain (MBD) that acts as a transcriptional repressor (e.g. (Lewis et al., 1992; Nan et al., 1997; Nan et al., 1996)). All MBD proteins, except MBD4, form complexes with histone-deacetylases (HDACs) (e.g. (Nan et al., 1998), reviewed in (Ballestar and Wolffe, 2001)). Methylation of promoter CpG islands leads to the binding of MBD proteins together with HDACs and other transcriptional repressors and, to a block of transcription initiation (Jones et al., 1998; Nan et al., 1998).

Mutations of the X-linked MeCP2 gene have been shown to result in Rett syndrome (Amir et al., 1999), an inherited neurodevelopmental disorder in females (Hagberg et al., 1983; Rett, 1966). In contrast to the initial publication (Tate et al., 1996), MeCP2 is not essential for embryonic development (Guy et al., 2001). At about three weeks of age, homozygous knockout mice start to show neurological deficiencies resembling symptoms of the Rett syndrome, including uncoordinated gait and reduced spontaneous movement. The phenotype of mice with brain-restricted deletion of MeCP2 is indistinguishable from that of MeCP2-deficient mice. $MeCP2^{+/-}$ females showed similar phenotypes although with delayed onset. It has been hypothesized that MeCP2 may not be essential for neurogenesis but is needed for the maintenance and stability of brain cells (Guy et al., 2001).

Normal and aberrant methylation patterns

As DNA methylation shows a tissue-restricted pattern, it has been postulated to play a role in the regulation of gene expression. Indeed, DNA binding of transcription factors like myc can be blocked by methylation of their binding site (Prendergast and Ziff, 1991). However, other transcription factors like Sp1 can bind to their recognition site even when CpG methylated (Harrington et al., 1988; Holler et al., 1988). In addition, CpG islands are unmethylated in expressing as well as non-expressing cells (see paragraph 'CpG islands').

While the link between transcriptional regulation and DNA methylation is unclear, DNA methylation plays undoubtedly an essential role in the definition and the maintenance of silenced euchromatic domains as well as in the maintenance of heterochromatic regions in the genome. The features of these two types of chromatin, euchromatin and heterochromatin are summarized in Table 1.

Placing a normally in euchromatin residing active gene into heterochromatin usually results in the silencing of the gene, although expression can be variegated (position effect variegation PEV), reviewed in (Dillon and Festenstein, 2002).

However, actively transcribed genes can also be found in heterochromatin (Sinclair et al., 2000). These heterochromatic genes seem to rely on their 'natural' heterochromatic environment for proper gene regulation (Eberl et al., 1993).

Examples of heterochromatin formation are X-chromosome inactivation and gene imprinting.

In female mammals, X-chromosome dosage compensation is achieved by the random silencing of one X chromosome. This silencing requires the expression of the untranslated Xist RNA from the affected chromosome that coats the chromosome in *cis* (Brown et al., 1991; Clemson et al., 1996; Marahrens et al., 1997; Penny et al., 1996). This coating results in secondary epigenetic marks such as DNA methylation and histone hypoacetylation, that are characteristic for the formation of silenced heterochromatin.

Parent-of-origin specific methylation marks control the expression of imprinted genes. This imprinted allele-specific methylation is established during late stages of germ cell development, maintained in the preimplantation embryo and is crucial for the later development of the embryo (reviewed in (Tilghman, 1999)). Imprinting and X-chromosome inactivation imply that methylation patterns are stably inherited. Nevertheless, the establishment of these epigenetic marks also requires a genome wide dynamic alteration of cytosine methylation patterns.

feature	**euchromatin**	**heterochromatin**
cytological appearance in interphase	dispersed	condensed, deeply stained
DNA sequence	predominantly unique	predominantly repetitive
gene density	high	low
replication timing	throughout S-phase	late S-phase
chromatin structure	accessible to nucleases	less accessible to nucleases
characteristic epigenetic modifications	histone hyperacetylation Lysine4 methylated in histone H3 Cytosine hypomethylation	histone hypoacetylation Lysine9 methylated in histone H3 Cytosine hypermethylation
gene activity **euchromatic gene** **heterochromatic gene**	 gene active or inducible gene silenced (variegated)	 gene silenced (variegated) gene active or inducible

Table 1: Characteristics of euchromatic and heterochromatic domains

Taken and adapted from (Richards and Elgin, 2002).

Genome wide reprogramming of methylation patterns after erasure of methylation marks occurs in developing germ cells and in preimplantation embryos (for review see (Reik et al., 2001)). Reprogramming of epigenetic information is not only crucial for normal development, but inappropriate epigenetic reprogramming has also been implicated in the variety of abnormalities that can be found in cloned embryos and animals derived from nuclear transfer (e.g. (Rideout et al., 2000), for review see (Rideout et al., 2001)).

Aberrant cytosine methylation patterns have also been found in many cancers (Robertson, 2002). Cancer cells often show a local hypermethylation of CpG islands (see Figure 1) that can lead to the silencing of the adjacent gene. If genes of cell cycle regulators or tumour suppressor genes are affected this can be causative for the development of cancer (Esteller, 2002). Paradoxically, cancer cells often show a concomitant genome wide hypomethylation besides local hypermethylation of some CpG islands (Ehrlich, 2002).

The zinc finger connection: a family portrait

The Sp/XKLF family of transcription factors contains a large number of proteins which, at the moment, include Sp1-Sp8 and 15 members of the krüppel-like factors (KLF) (Bouwman and Philipsen, 2002). Members of the family have a characteristic feature: three conserved zinc fingers of the Cys2His2 type as DNA-binding domain (for review see (Philipsen and Suske, 1999)) that recognize with varying affinities the same regulatory sequences known as GC-[GGGGCGGGG] and GT/CACC-[GGTGTGGGG] boxes (Gidoni et al., 1985; Giglioni et al., 1989; Imataka et al., 1992; Letovsky and Dynan, 1989). Each zinc finger is thought to contribute with three DNA-contacting amino acids (Fairall et al., 1993; Pavletich and Pabo, 1991) to the DNA binding properties of the family members. DNA-contacting amino acids are KHA for the first (KLA for Sp2), RER for the second and RHK (alternatively RHL) for the third zinc finger (Philipsen and Suske, 1999).

These G-rich DNA sequences (commonly referred to as ´Sp1-binding sites´, according to the first identified member of the transcription factor family binding to them) are found in promoters and enhancers of house-keeping genes as well as many tissue-specific, viral and cell cycle regulating genes.

Besides their importance for direct transcriptional activation, it has been shown for the APRT gene that these DNA motifs are involved in the maintenance of the methylation-free status of CpG islands (Brandeis et al., 1994; Macleod et al., 1994).

As the name indicates, the factor family Sp/XKLF can be subdivided due to possession of certain N-terminal domains apart from the zinc finger domain and their chromosomal

locations (reviewed in (Bouwman and Philipsen, 2002; Philipsen and Suske, 1999). The following overview concentrates on the Sp-factors.

Structural and biochemical features of the Sp family

Sp1 is the founding member of the Sp family. Its discovery dates back to the early 1980´s, being identified as a promoter-specific factor that binds to the SV40 early promoter (Dynan and Tjian, 1983a; Dynan and Tjian, 1983b). The name of Sp1 originates from the purification protocol, which used **s**ephacryl- and **p**hosphocellulose-columns as last purification steps. Nowadays Sp is also referred to as **s**pecificity **p**rotein.

One characteristic feature of the known eight Sp factors is their co-localisation with a HOX gene cluster, indicating the close evolutionary relationship of these family members. Sp1 and Sp7 co-localise on 12q13.13 with HOX C, Sp2 and Sp6 on 17q21.31/32 with HOX B, Sp3 and Sp5 on 2q31.1 with HOX D and Sp4 and Sp8 on 7p21.2 with HOX A (see reviews (Bouwman and Philipsen, 2002; Philipsen and Suske, 1999) and references therein).
The second characteristic of Sp factors is their possession of two protein domains the functions of which are still elusive (see Figure 3).
The Buttonhead box (Btd box) consists of 11 conserved amino acids first described in the Sp1 homologue Buttonhead of *Drosophila* (Wimmer et al., 1993). In the Sp factors it resides directly N-terminal to the zinc finger domain. A deletion of the highly charged domain C (Courey and Tjian, 1988) which comprises the Btd box results in a reduced transactivation potential of Sp1 *in vitro*. In addition, the Btd box of Sp1 or Sp3 is important for synergistic activation of sterol-regulatory element-binding proteins (SREBP) at promoters that only have one SREBP binding site, whereas it is dispensable at promoters including more than one SREBP binding site (Athanikar et al., 1997; Yieh et al., 1995).
The Sp box is located at the N-terminus of the proteins (Harrison et al., 2000) and may have a function in the regulation of proteolysis of Sp factors. It not only contains an endoproteolytic cleavage site but also overlaps partially with the N-terminal 54 amino acids of Sp1 that target proteasome-dependent degradation *in vitro* (Su et al., 1999).
Most of the Sp family members contain a PEST sequence at varying positions in the protein. The PEST domain (reviewed in (Rechsteiner and Rogers, 1996)) has been reported for a number of proteins that show inducible degradation.

The alignment of the Sp proteins shows that they can be subdivided further, as Sp1-4 contain a characteristic alternating arrangement of glutamine-rich activation domains and serine/threonine-rich domains, whereas Sp5-8 do not show this arrangement and are remarkably shorter.

Sp1

Using a co-transfection assay involving *Drosophila* SL2 cells (Courey and Tjian, 1988), as well as a variety of *in vitro* assays (Kadonaga et al., 1987; Kadonaga et al., 1988) the functional domains of Sp1 were mapped and characterised. Both glutamine-rich regions (domains A and B in Figure 3) can act as strong activation domains (Courey and Tjian, 1988). It has been shown that contact between Sp1 and the TBP-associated factor dTAFII110 (necessary for transcriptional activation by Sp1) requires the hydrophobic amino acids within Sp1 domain B but not the glutamine residues (Gill et al., 1994). Similarly, the exchange of a hydrophobic phenylalanine residue with a hydrophilic amino acid in a glutamine-rich region of the hTAFII130 compromises its ability to associate with domain B of Sp1 (Rojo-Niersbach et al., 1999).

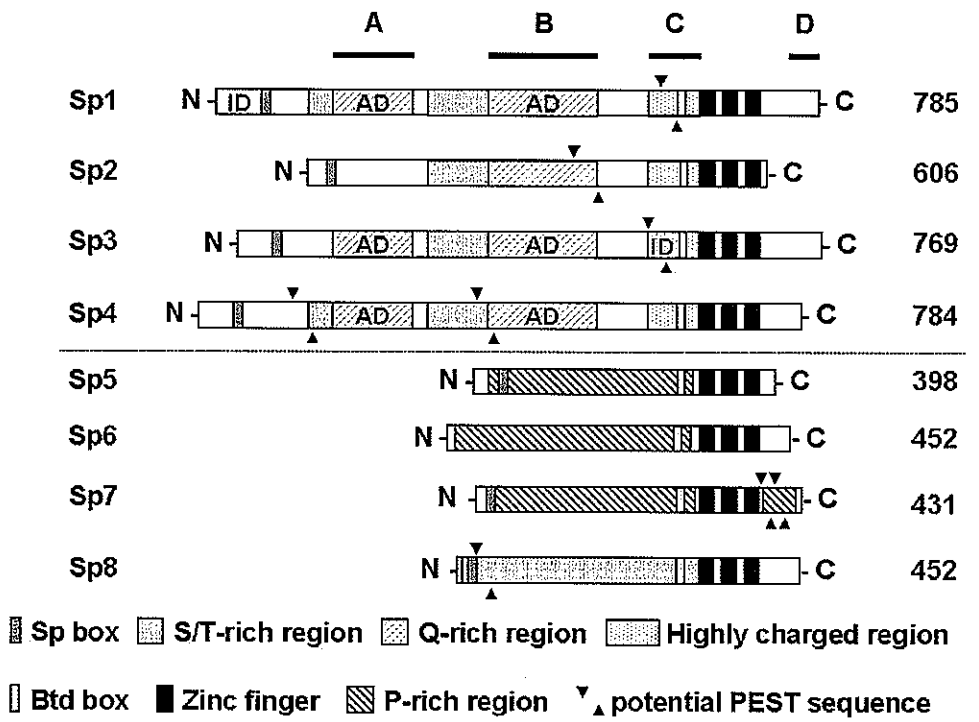

Fig. 3: Structural motifs of Sp factors.
A, B, C, and D modules are marked with black bars as first described for Sp1 in (Courey, 1988). Pairs of arrow heads point at PEST domains. Activation (AD) and inhibitory (ID) domains are depicted. S/T, serine/threonine, Q, glutamine, P, proline. The length of the proteins is indicated in amino acid number at the right. For further explanations see main text. Taken from (Bouwman and Philipsen, 2002).

In vivo studies have shown that Sp1 bound to distal enhancer regions can interact with Sp1 bound at sites proximal to the promoter and synergistically activate transcription (Courey et al., 1989). *In vitro* data indicate that this synergistic activation is mediated by Sp1 tetramers bound to proximal and distal sites that form multiple stacked tetramers (Mastrangelo et al., 1991), thereby looping out the intervening DNA (Li et al., 1991; Mastrangelo et al., 1991; Su et al., 1991).

For short- as well as long-distant synergism, the glutamine-rich domains A and B and the C-terminal domain D are essential (Pascal and Tjian, 1991). Domain B seems to be crucial for the formation of homomultimeric complexes (Pascal and Tjian, 1991).

The phenomenon of superactivation has also been described for Sp1 (Courey et al., 1989). Here, non-DNA-binding forms of Sp1 (superactivator) can enhance transcriptional activation by DNA-binding forms (activator). For efficient superactivation, the superactivator needs the two domains A and B, whereas the main function of the activator is to tether the superactivator to the DNA. The zinc finger domain and part of the B domain seem to fulfil this purpose (Courey et al., 1989; Pascal and Tjian, 1991).

Sp1 co-regulators

As partially purified human or *Drosophila* TFIID fractions contained unknown proteins necessary for efficient transcriptional activation by Sp1 (Pugh and Tjian, 1990), the existence of co-activators was proposed.

The TAFII subunits of the transcription factor complex TFIID are one class of co-activators. *In vitro* studies showed the direct interaction of the glutamine-rich activation domains of Sp1 with the hTAFII130 (Rojo-Niersbach et al., 1999; Tanese et al., 1996) and its *Drosophila* homologue dTAFII110 (Gill et al., 1994; Hoey et al., 1993) as well as with the TATA-box binding-protein TBP from the TFIID complex (Emili et al., 1994).

Another multi-subunit co-activator complex CRSP (cofactor required for Sp1) has been recently described in human (Ryu et al., 1999) as being required for Sp1 activation on naked DNA. CRSP represents a new cofactor complex for Sp1 that shares some subunits with other cofactors but also contains some unique subunits (Ryu and Tjian, 1999).

The human multi-subunit cofactor ARC (activator-recruited cofactor) functions on chromatin templates to mediate synergistic gene activation by the transcriptional activators SREBP-1a and Sp1 (Naar et al., 1998). ARC can directly interact with different activators like SREBP-1a, VP16 and the p65 subunit of NF-κB and thereby strongly enhance transcription *in vitro* on chromatin-assembled DNA templates (Naar et al., 1999). Recently, it has been shown that ARC consists of two multi-subunit complexes: a large transcriptionally inactive complex ARC-L (ARC240, ARC250, cdk8 and cyclinC) and the smaller transcriptionally highly active CRSP complex (Taatjes et al., 2002). Furthermore, it has been shown that the different subunits of

CRSP are also targeted by different transcription activators (Boyer et al., 1999; Ito et al., 1999; Rachez et al., 1999). Structural data suggest that CRSP undergoes dramatic activator-specific conformational changes upon binding of the activator. The model implicates a modulating effect of transcriptional activity by the ACR-L-specific subunits (Taatjes et al., 2002).

An example for negative regulation is the complex formation with the transcriptional co-repressor histone deacetylase 1 (HDAC1). Direct interaction of Sp1 (or Sp3) with HDAC1 via the carboxy-terminal domain (Doetzlhofer et al., 1999) has been reported to mediate Sp1-dependent repression of transcription.

Sp2

Sp2 was identified by screening a human cDNA library with the Sp1 zinc finger domain at low stringency (Kingsley and Winoto, 1992). It differs from Sp1, Sp3 and Sp4 by several features. First, it has different binding specificities, as *in vitro* it only binds weakly to the GT box of the T-cell antigen receptor α (TCRα) promoter and not at all to the GC box therein. This can easily be explained by homology comparisons between Sp factors. The homology to Sp1 at the zinc finger domain is only 72%, compared to 90% for Sp3 (Kingsley and Winoto, 1992). Most importantly, a leucine residue substitutes the histidine residue, one of three amino acids in the first zinc finger that contact the DNA. Furthermore, Sp2 contains only one glutamine-rich region and the C-terminal domain D is also missing. Hence, it is likely that Sp2 shows different activation characteristics than Sp1, Sp3 and Sp4 as domains known to be essential for synergism and superactivation are missing (Pascal and Tjian, 1991).

Only very few reports so far could implicate a regulatory role for Sp2 in gene transcription. It has been shown that Sp2 and Sp3 (as well as KLF-6) bind to regulatory elements in the intron of the collagen-specific molecular chaperone HSP47 (Yasuda et al., 2002). Another report suggests a cell-type dependent role of Sp2, as in mammalian C3H10T1/2 cells it activates a construct containing the mouse CTP:phosphocholine cytidylyltransferase α promoter, whereas in *Drosophila* SL2 cells it represses Sp1- and Sp3-activated transcription of the same construct (Bakovic et al., 2000).

Sp3

The Sp3 protein shows a striking homology to Sp1 (Hagen et al., 1992; Kingsley and Winoto, 1992). Consequently, its binding specificities for GC and GT boxes are almost undistinguishable from that of Sp1. Nevertheless, functional analysis revealed the many faces of Sp3 as a regulator whose activity is dependent on both the promoter and the cellular context.

Sp3 has been shown to activate transcription in many cell lines similar to Sp1 (e.g. (Cogan et al., 2002; Galvagni et al., 2001; Ihn and Trojanowska, 1997; Kishikawa et al., 2002; Udvadia et al., 1995; Zhao and Chang, 1997)).
Other reports showed Sp3 to be inactive or only weakly activating (e.g. (Bahouth et al., 2002; Hagen et al., 1994; Kumar and Butler, 1997)). Co-transfection experiments showed that Sp3 and Sp1 can activate transcription in an additive (Ihn and Trojanowska, 1997; Jiang et al., 1997; Ko et al., 1998; Teunissen et al., 2002) or a synergistic (Bigger et al., 1997; Castoldi and Chu, 2002; Netzker et al., 1997) manner.

Due to their similar binding specificities, Sp1 and Sp3 compete for the same binding sites. At promoters containing multiple adjacent binding sites, Sp3 can act as a transcriptional repressor (Birnbaum et al., 1995; Chadjichristos et al., 2002; Dennig et al., 1996; Majello et al., 1997).
The repressive activity of Sp3 has been mapped to an inhibitory domain, which is located between the second glutamine-rich region and the DNA binding domain (Dennig et al., 1996). The inhibitory domain even exerts its effects when connected to heterologous activation domains and was shown to be absolutely dependent on the amino acid triplet KEE (Dennig et al., 1996). Post-translational modifications of this amino acid triplet have been shown to contribute to its inhibitory function and are described in more detail in the paragraph 'post-transcriptional modifications of Sp family members'.
As Sp3 exists in three isoforms, one full-length protein of about 110 kDa, and two shorter species of about 60-70 kDa (arising from alternative translation initiation from internal AUG codons) (Kennett et al., 1997), analysis of its transcriptional activities has further been complicated.
Internally initiated Sp3 proteins lack the first glutamine-rich activation domain and have therefore been suggested to act as potent inhibitors of Sp1/Sp3-mediated activation by competition for binding sites (Kennett et al., 1997; Pan et al., 2000; Whetstine and Matherly, 2001). Nevertheless, inhibitory properties of Sp3 cannot be solely ascribed to the short forms, as full-length Sp3 can repress Sp1-mediated transcription as well (Dennig et al., 1996; Fandos et al., 1999).

Sp4

Sp4 was cloned by recognition site screening using the GT-box of the uteroglobin promoter (Hagen et al., 1992). Consistent with the highly conserved DNA binding domain, Sp4 has the same DNA binding specificity than Sp1 and Sp3. However, functional analysis revealed that Sp4, unlike Sp1, is not able to act synergistically through adjacent binding sites. Nevertheless, it can be subject to Sp1-mediated superactivation and Sp3-mediated

repression (Hagen et al., 1995). Transfection studies in *Drosophila* SL2 cells and mammalian cell lines showed some promoter to be Sp4-responsive (Ahlgren et al., 1999; Hagen et al., 1995; Hagen et al., 1994; Wong et al., 2001), while others could only be activated by Sp1 and Sp3 (Bigger et al., 1997; Yan et al., 2000).

In vivo functions of Sp proteins

Due to their similar or even identical DNA sequence specificities and the often concomitant expression of several family members in the same cell, possible redundancies and interferences between members of this gene family in *in vivo* experiments, including targeted gene inactivation, should be taken into account.

Sp1

The results of an extensive number of investigations have implicated Sp1 not only in the regulation of a huge number of housekeeping genes but also in the regulation of tissue-specific genes. Sp1 has also been assigned a role in nuclear processes like the maintenance of methylation-free islands (Brandeis et al., 1994; Macleod et al., 1994), cell cycle regulation (Birnbaum et al., 1995; Datta et al., 1995; Karlseder et al., 1996; Lin et al., 1996) and chromatin remodelling (Ellis et al., 1996; Jongstra et al., 1984; Philipsen et al., 1993). Very recently, it has been demonstrated that the glutamine-rich domains of Sp1 exhibit a strong boundary activity in yeast, when tethered to DNA (Ishii and Laemmli, 2003). This boundary activity preserved a gene from silencing when interposed between the gene and a silencing source.

Sp1 is an abundant transcription factor in many, if not all, mammalian cell types. Nevertheless, its expression level varies between different cell types and changes during development and differentiation, with fully differentiated cells often showing reduced Sp1 expression (Saffer et al., 1991).

Surprisingly, despite its ubiquitous expression and its involvement in essential cellular processes, Sp1 is dispensable in murine ES cells (Marin et al., 1997). Sp1-deficient ES cells are viable, grow normally and can be readily induced to differentiate *in vitro* into embryoid bodies. Only when challenged by the cellular demands of early embryonic development do Sp1-deficient cells show insufficiencies. Sp1-deficient embryos are severely retarded in development, die around day 10 of gestation and show a broad range of abnormalities. Some embryos show differentiated structures like developing eye and heart, otic vesicles, somites, erythroid cells and extra-embryonic tissues while others resemble undifferentiated cell clumps. The cellular defect of Sp1-deficiency is cell-autonomous as revealed by injection of Sp1-deficient ES cells into wildtype blastocysts. Resulting chimeric embryos show high

contributions of Sp1-deficient cells to all embryonic tissues during early development, with a rapid decline around day 10 and no detectable contribution to any tissue of newborn mice. Despite the large number of putative Sp1 target genes found in cell culture transfection experiments, only two genes tested showed reduced expression levels in Sp1-deficient embryos, thymidine kinase and methyl-CpG binding protein 2 (MeCP2).

Sp2

For this family member no *in vivo* data are available. Even *in vitro* expression data are restricted to some reports on Sp2 expressing cell lines (Bigger et al., 1997; Halim et al., 2001; Kingsley and Winoto, 1992) and rat brain (Bigger et al., 1997).

Sp3

As Sp1, Sp3 is widely expressed (Hagen et al., 1992). Although Sp1 and Sp3 show similar, if not identical, binding specificities (Hagen et al., 1992) and usually are co-expressed in mammalian cells, the Sp3 knockout in mice revealed distinct and highly specific functions of Sp3 in late developmental stages (Bouwman et al., 2000). Therefore, the probable redundant and overlapping functions of these two factors in the regulation of many genes are incomplete. Sp3-deficient embryos are growth retarded, but survive during embryonic stages without showing gross defects other than a reduced body weight. But newborn Sp3-deficient mice invariably die within minutes after birth due to respiratory failure. Histological analysis of the lung only showed minor alterations. Expression of superfactant proteins as indicators of lung development was normal. Histological analysis of other organs revealed a pronounced defect in late tooth development in Sp3 deficient mice. The development of the dentin/enamel layer is impaired in these mice due to a strongly reduced expression of ameloblast-specific gene products.
Further investigation indicated that the severity of the Sp3 deficient phenotype is dependent on the genetic background, as it increases with increasing C57Bl/6 contribution to the genome, resulting in prenatal lethality (Bouwman, 2002). It has been proposed that the improper heart development observed in these embryos also might contribute to the postnatal lethality on the mixed 129/C57Bl/6 background described before.

Sp4

Compared to other Sp factors, Sp4 shows a restricted expression pattern, with predominant expression in the brain (Hagen et al., 1992). It is also detectable in other tissues like epithelial tissue, testis and developing teeth (Gollner et al., 2001; Supp et al., 1996). Sp4 knock outs were done either by deleting the zinc finger domain within a 19 kb deletion (Supp et al., 1996) or by deleting the exons encoding for the N-terminal activation domains (Gollner

et al., 2001). Nevertheless both approaches resulted in similar phenotypes. Sp4 deficient pups were born at the expected Mendelian ratio and showed no obvious abnormalities. However, two thirds of them died within four weeks after birth. The cause of death remains unknown. Surviving Sp4-deficient mice were growth retarded and showed severe impairment in their reproduction. Adult Sp4-deficient males did not breed at all, although spermatogenesis seemed to be normal. As Sp4 expression is predominant in the brain, neurological defects resulting in abnormal mating behaviour were implicated as possible reasons for the observed impaired reproduction. However, histological examinations of the hypothalamus and the vomeronasal organ as important organs for reproductive behaviour (Buck, 1995) did not show gross abnormalities. In addition, two pheromone receptors showed normal expression in the vomeronasal organ of Sp4-deficient newborn mice. Sp4-deficient females showed a dramatic decrease in uteri size, a late onset of puberty and only sporadical mating success (Gollner et al., 2001).

Sp5

Sp5 shows a highly dynamic expression pattern during mouse embryogenesis in the developing brain, the spinal cord, the trigeminal ganglia, the somites and additional sites outside the nervous system (Treichel et al., 2001). However, mice homozygous for a targeted mutation in Sp5 show no obvious phenotype (Harrison et al., 2000).

Sp6

Sp6 is widely expressed (Scohy et al., 2000), However, to date no functional analysis has been published.

Sp7

Sp7, also called osterix, shows a tissue-specific expression in all developing bones. Sp7-deficient mice lack bone formation due to a defect in osteoblast differentiation (Nakashima et al., 2002).

Sp8

Sp8 has recently been described (Bouwman and Philipsen, 2002). Neither expression pattern nor function has been studied yet. However, *in silico* analysis suggests that the main expression sites are the prostate and the brain (S. Philipsen, unpublished data).

Non-mammalian relatives of the Sp family

The *Drosophila* gene buttonhead (btd) encodes a zinc finger protein that is structurally and functionally related to the mammalian transcription factor Sp1 (Wimmer et al., 1993). Btd has been characterized as a head gap gene, since it is expressed in the syncytial blastoderm embryo in a stripe covering the anlagen of the antennal, intercalary and mandibular head segments. In btd mutant embryos these head segments are deleted (Cohen and Jurgens, 1990). Mammalian Sp1 and *Drosophila* BTD have been shown to interact with the same TATA box-binding protein-associated factors and thereby, support transcriptional activation *in vitro* (Schock et al., 1999b). A human Sp1 transgene under the control of btd-regulatory elements is able to rescue the btd phenotype partially in that it restores the development of the mandibular segment (Wimmer et al., 1993). Apart from its role in head segmentation in the early *Drosophila* embryo, btd expression in the developing brain and the forming peripheral nervous system of the later embryonic stages, suggest its involvement in a number of developmental processes (Wimmer et al., 1996).

A second Sp1-like *Drosophila* gene, termed *Drosophila* Sp1 (D-Sp1) has been identified (Wimmer et al., 1996). D-Sp1 and btd show not only similar expression patterns during postblastodermal embryogenesis but also a close sequence relationship of the zinc finger region and shared cytological location of the two genes. Therefore, it has been proposed that they act as a gene pair with at least partially redundant neural functions (Schock et al., 1999a; Wimmer et al., 1996). No D-Sp1 expression can be detected at the blastoderm stage, but its transgene expression can, similar to the forced expression of human Sp1, rescue the mandibular defect of the btd phenotype. The common expression domains during postblastodermal development include a restricted pattern in the developing peripheral and central nervous system and embryonic brain, as well as in the leg anlagen.

Despite the existence of *Drosophila* Sp1 homologues, Schneider line 2 (SL2) cells (Schneider, 1972), originating from late stages of *Drosophila* embryos, are routinely used as a host system for the functional analysis of Sp transcription factors. DNA binding activity of Sp factors seems to be absent from SL2 cell extracts (Courey and Tjian, 1988; Santoro et al., 1988).

Post-transcriptional modifications of Sp family members

The most important post-translational modifications of proteins include phosphorylation, glycosylation and acetylation. Most of the data generated concerning protein modifications, concentrate on Sp1. Nevertheless, other Sp family members are likely to be processed in similar ways. The plethora of different modifications and different modifying proteins,

themselves regulated by physiological and environmental signals suggests a role for post-transcriptional modifications in fine-tuning the activity of the Sp-family.

Phosphorylation

Protein phosphorylation is known to play a key role in many signal transduction pathways and in modulating the activity of sequence-specific transcription factors. Sp1 has been shown to be a phosphoprotein and a target for multiple cellular kinases. Different kinases phosphorylate different amino acid residues in different parts of Sp1. Sp1 phosphorylation can decrease or increase its DNA binding activity, implicating influences on transcriptional activation properties. Interestingly, Sp1 phosphorylation might to play a role in growth and cell cycle regulation as well as cellular differentiation.

The two electrophoretic variants of Sp1 (95kDa and 105kDa) in human cells have been ascribed to two differentially phosphorylated protein forms (Jackson et al., 1990). The first characterized Sp1-kinase was DNA-dependent protein kinase (DNA-PK) (Gottlieb and Jackson, 1993; Jackson et al., 1990). Efficient phosphorylation of Sp1 by DNA-PK depends on its DNA-binding activity, although the sites of modification lie outside the DNA-binding domain. Primarily the serine residues within the transactivation domain are the target sites for DNA-PK. DNA binding characteristics of Sp1 were not influenced by DNA-PK-mediated phosphorylation. The physiological significance of Sp1 phosphorylation by DNA-PK remains unclear.

Compared to undifferentiated cells, phosphorylation of Sp1 is increased in differentiated liver cells, and has been correlated to a decreased DNA binding activity (Leggett et al., 1995). Casein kinase II has been shown to be able to phosphorylate the C-terminus of Sp1. Phosphorylation of a threonine in the second zinc finger by casein kinase II leads to a concomitant decrease in DNA binding activity (Armstrong et al., 1997).

Phosphorylation by cAMP-dependent protein kinase A (PKA) *in vitro* increases Sp1 activity, at least partially through enhancing its DNA-binding (Rohlff et al., 1997).

A not yet further characterized kinase has been shown to phosphorylate the C-terminal part of Sp1 in a cell-cycle dependent manner *in vivo*, with enhanced phosphorylation in late G1 phase (Black et al., 1999).

PKC-ς has been shown to be able to phosphorylate the zinc finger region of Sp1 (Pal et al., 1998). PKC-ς-mediated phosphorylation of Sp1 has been implicated in the induced expression of growth factors like vascular endothelial growth factor (VEGF) in renal cell carcinoma (Pal et al., 1998) and platelet-derived growth factor β-chain (PDGF-β) in smooth muscle cells (SMC) (Pal et al., 1998). Phosphorylation of Sp1 in smooth muscle cells has

been implicated with increased expression of the death agonist Fas ligand (FasL) leading to increased SMC apoptosis (Kavurma et al., 2001).

Phosphorylation of at least one of the two linker regions connecting the three zinc fingers of Sp1 is occurring during G2/M phase of the cell cycle (Dovat et al., 2002). The kinase responsible for this phosphorylation so far is unknown. Nevertheless, phosphorylation in the highly conserved linker regions of Cys2His2 proteins has been proposed to mediate their mitotic inactivation (Dovat et al., 2002). Indeed, Sp1 shows reduced DNA binding activity in mitotic extracts and is displaced from mitotic chromatin (Martinez-Balbas et al., 1995).

Recently it has been shown that cyclinA-CDK complex can phosphorylate Sp1 on a serine residue within the N-terminal region. This phosphorylation enhances DNA binding of Sp1 and stimulates transcription from Sp1-regulated promoters (Fojas de Borja et al., 2001; Haidweger et al., 2001). Since expression of cyclinA-cdk2 and cyclinA-cdc2 complexes is strictly regulated during cell cycle, this phosphorylation might play a role in the growth and cell cycle regulation of transcription.

Glycosylation

The covalent O-linked monosaccharide N-acetylglucosamine (GlcNAc) is an abundant post-translational modification found on nuclear and cytoplasmic proteins. Sp1 contains multiple O-linked GlcNAc-residues on serines and threonines, the majority of which lie within the N-terminal half of the protein (Jackson and Tjian, 1988).

Glycosylation of Sp1 has been linked to regulatory functions in self-association, interaction with basal transcription factors like TAFII110 (Roos et al., 1997) and modulation of its degradation (Han and Kudlow, 1997). It has been shown that an O-GlcNAc glycosylation of a Sp1 peptide containing part of the B domain inhibits the interaction between the peptide with Sp1 itself or with TAFII110. The inhibition of hydrophobic interactions between proteins by O-GlcNAc has been proposed to prevent untimely and ectopic protein-protein interactions (Roos et al., 1997). Indeed, overexpression of O-GlcNAc transferase (OGT) inhibits Sp1-driven transcription (Yang et al., 2001). Recently, direct interaction between OGT and the co-repressor mSin3A and histone deacetylase HDAC1 has been shown to repress Sp1-driven, as well as basal, transcription (Yang et al., 2002). Interestingly, glycosylation and phosphorylation seem to be reciprocally correlated. It has been shown that an increase in glycosylation on Sp1 leads to a decrease in its level of phosphorylation (Haltiwanger et al., 1998). Reduced O-glycosylation (Han and Kudlow, 1997) as well as increased phosphorylation (Mortensen et al., 1997) of Sp1 are associated with increased proteasome-susceptibility. Glycosylation of Sp1 may play a role in the coordination of cell growth and nutritional environment, since in cells that are stressed with glucose deprivation and adenylate cyclase activation hypoglycosylated Sp1 undergoes proteasome-dependent

degradation (Han and Kudlow, 1997). Interestingly, direct interaction of Sp1 with the ATPase subunit (Sug1/p45) of the 26S proteasome could be shown to play an important role in the proteasome-dependent degradation of Sp1 (Su et al., 2000).

Acetylation of Sp3

The inhibitory domain of Sp3 lies between the second glutamine-rich activation domain and the DNA-binding domain and has been shown to suppress transcriptional activation mediated by the glutamine-rich activation domains. The amino acid triplet KEE within this domain is essential for its inhibitory effects on transactivation (Dennig et al., 1996). Furthermore the ε-amino group of the lysine residue within this KEE triplet is a target for acetylation. Mutation of the lysine rendered the Sp3 protein inert to acetylation, with a resulting increase in its ability to activate transcription (Braun et al., 2001).

SUMOylation of Sp3

The very same lysine residue of the KEE triplet that is essential for acetylation has very recently been reported to be the target for another post-transcriptional modification, the SUMOylation (Sapetschnig et al., 2002). SUMO-proteins are small ubiquitin-like proteins that can be covalently linked to the ε-amino group of lysine residues (for review see (Muller et al., 2001) and references therein). Several other proteins have been shown to be modified by SUMOylation (e.g. p53 (Gostissa et al., 1999; Rodriguez et al., 1999). The biological consequences of SUMOylation seem to depend on the target, suggesting roles in protein-association, subcellular localization, increasing as well as decreasing transactivation potentials of transcription factors. For Sp3, SUMO conjugation mediated by PIAS1 (protein inhibitor of activated STAT1) to the lysine residue results in silencing of its activity. Silencing obviously does not inhibit DNA binding. In addition, sumoylated Sp3 is sequestered in the nuclear periphery (Ross et al., 2002). Modulation of subnuclear localization could indeed impose another level of transcriptional regulation.

As acetylation and SUMOylation target the same amino acid of Sp3 modification of this amino acid appears to be crucial for Sp3 activity.

<u>Deregulation of Sp factors and disease</u>

Gene targeting studies in mice have shown that a functional deletion of Sp1 and Sp3 is incompatible with embryonic or postnatal survival, respectively (Bouwman et al., 2000; Marin et al., 1997). Therefore, it is not surprising that no clinically manifested disease could be ascribed to deleterious mutations in these genes. Nevertheless, several diseases have been linked to naturally occurring polymorphisms or mutations in 'Sp1-binding sites' of regulatory region of genes that render them either less or more susceptible to Sp1-mediated

transcriptional activation. It should be noted that although Sp1 has been directly addressed in these studies as the factor that regulates the transcription of these genes, it cannot be excluded that other Sp/XKLF family members also exert transcriptional activation by binding to these 'Sp1-binding sites'.

Substitution of a single base in the proximal Sp1 site within the promoter of the human low density lipoprotein (LDL) receptor has been implicated with a typical type of familial hypercholesterolemia (Koivisto et al., 1994). A polymorphic Sp1 binding site in the collagen typeIα1 gene has been correlated with reduced bone density and osteoporosis (Grant et al., 1996; Uitterlinden et al., 1998). In the human retinoblastoma gene, a point mutation within a Sp1-binding site interferes with its binding and is associated with hereditary retinoblastoma (Sakai et al., 1991). A point mutation disrupting a Sp1 binding site in the promoter of factor VII has been identified as one cause for factor VII deficiency and a concomitant severe bleeding disorder (Carew et al., 1998). The importance of a 'Sp1 binding site' in the promoter of the collagen type VII gene for its expression has been shown by a C to A transition which abolishes transcription and is implicated in hereditary dystrophic epidermolysis bullosa (Gardella et al., 2000).

Besides reduced gene expression due to reduced Sp1 binding to its recognition site, opposite effects have been observed, where a site mutation leads to increased binding and therefore, increased transcriptional activation.

Increased Sp1 binding and therefore increased expression of a γ globin gene due to a mutation in its promoter could be a cause of hereditary persistence of fetal globin (Ronchi et al., 1989; Sykes and Kaufman, 1990). A base pair exchange resulting in a stronger Sp1 binding site and increased expression of myeloperoxidase has been correlated with increased risks for subsets of myeloid leukemias (Reynolds et al., 1997).

Some data suggest that altered levels of Sp factors contribute to deregulated gene expression and disease. Elevated Sp1 expression may result in up-regulated expression of degradative enzymes and down-regulated expression of protease inhibitors, which have been demonstrated in keratoconus, a cornea-thinning disease (Whitelock et al., 1997). Chapter 5 of this thesis describes an increased incidence of ocular abnormalities in mice heterozygous for Sp1.

Expression of the mutated form of the protein huntingtin results in the inherited neurodegenerative disorder of Huntington's disease and is manifested by progressive cognitive, psychiatric and motor symptoms, leading to death. In contrast to the normal huntingtin gene, the mutated gene contains an expansion of CAG trinucleotide repeats, resulting in an expanded stretch of polyglutamine in the mutant huntingtin protein (reviewed in (Cummings and Zoghbi, 2000; Fischbeck, 2001; Ross, 2002)). The length of the glutamine

extension correlates with the age of onset of Huntington's disease. The cellular function of normal huntingtin stays elusive.

It has been suggested that polyglutamine expansions result in aberrant interactions with nuclear proteins thereby corrupting normal transcription in neurons. Recently, it has been shown that mutant huntingtin interacts with Sp1 via these glutamine stretches (Li et al., 2002). Furthermore, mutant huntingtin specifically disrupts the normal interaction of the Sp1 with its co-activator TFII130, resulting in decreased expression of Sp1-dependent neuronal genes like the dopamine D2 receptor gene (Dunah et al., 2002). The deregulated expression of Sp1 downstream genes may be an early step in the neurodegenerative process resulting in Huntington's disease.

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Chapter 2

Sp1 target genes in ES cells: detection and analysis

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Abstract

Sp1 is a well-known mammalian transcription factor that has been implicated in the regulation of hundreds of genes. To characterize the role of Sp1 in transcriptional regulation in ES cells, we analysed gene expression profiles of mouse embryonic stem cells deficient in Sp1 using DNA microarray technology. Surprisingly, far less genes than expected showed significant changes in expression levels. From the genes that were tested by Northern blot, cytokeratin19 and 14-3-3σ could be verified as genes that are down-regulated in Sp1-deficient ES cells. The level of down-regulation however proved for both genes to be less than what has been determined by the microarray analysis. Methylation-sensitive Southern blot analysis of the cytokeratin19 promoter did not show altered methylation patterns of HpaII/MspI sites in Sp1 heterozygous or homozygous knockout ES cells.

Introduction

Sp1-binding sites are found in regulatory regions of numerous genes. In transient transfection assays Sp1 has indeed been shown to activate transcription of many reporter genes via binding to this regulatory regions. However, transcriptional activation of target genes that are found within the chromatin context of cells usually imposes higher demands on the transcriptional activator than activation of genes on naked DNA molecules. To characterize the role of Sp1 in transcriptional regulation in the cellular context, we analysed gene expression profiles of ES cells with targeted deletion of the full-length Sp1 gene. For this aim, we have applied the microarray technology for the comparison of RNA from Sp1 heterozygous and Sp1 homozygous knockout ES cells.

DNA microarrays have become a powerful technology that enables the analysis of expression profiles of thousands of genes, sequences of which are fixed on the chip. Total or polyA+ RNAs are isolated from usually two populations of cells to be compared. Hybridisation samples for Affymetrix chips are usually prepared by annealing a T7-RNA-polymerase promoter sequence linked to a poly-d(T) deoxyoligonucleotide to a mRNA population. First-strand cDNA synthesis is done by use of reverse transcriptase, followed by a second-strand synthesis. Subsequently, T7 mRNA polymerase produces cRNA from the cDNA templates, incorporating biotin-conjugated ribonucleotides. Biotin-cRNA populations are fragmented by heat, hybridised to the DNA chip and stained by a streptavidin-linked fluorescent dye. Computer-based analysis detects differences in fluorescence-intensities from different samples.

Application of this technology for the detection of Sp1 target genes in ES cells yielded an astonishing small number of consistently differentially regulated genes. We were interested in the known genes and so far verified down-regulation of cytokeratin19 and 14-3-3σ by Northern blot analysis.

Results

Differential gene expression in Sp1-deficient ES cells detected by microarray analysis

To identify the influence of loss of Sp1 function, we compared the gene expression in two independent cell lines each of Sp1 heterozygous knockout ES cells and Sp1 homozygous knockout ES cells. Sp1 knockout alleles had been generated by deletion of the C-terminal zinc finger binding domain of the Sp1 gene (Marin et al., 1997). Amplified mRNA from these cells was hybridised to oligonucleotide arrays either representing 11000 (11k) or 19000 (19k) gene segments (Affymetrix).

In order to increase the reliability of the results we performed hybridisations using two pairs of independently generated $Sp1^{+/-}$ and $Sp1^{-/-}$ ES cells. Both pairs of RNAs were applied to both oligonucleotide arrays. In experiment 1 mRNA from clone $Sp1^{+/-}$20/8 was compared with mRNA from clone $Sp1^{-/-}$19 using the 19k chips, in experiment 2 clone $Sp1^{+/-}$11 was compared with clone $Sp1^{-/-}$20 using 11k and 19k chips. For experiment 3 new cRNA was prepared from the mRNA batch used in experiment 1 and applied on 11k and 19k chips. A list of differentially expressed genes and expressed sequence tags (ESTs) from the TIGR database of unknown genes is given in Table 1. Only those genes and ESTs are listed that showed an average difference in expression level between $Sp1^{+/-}$ and $Sp1^{-/-}$ cells of at least a factor 3. Although, due to this selection criterium, the factors of down-regulation are significant, differences are detectable. For example the gene 14-3-3σ (also called stratifin) shows similar down-regulation in Sp1-deficient cells for experiment 2 and 3 on the 11k chips (factor 16), whereas on the 19k chips down-regulation for this gene (here called stratifin) for the same experiments is much less (factor 4). However, the first hybridisation of the 19k had given a factor 13, comparable to the results from the 11k chips.

Remarkably, a number of detected genes that are according to the microarray analysis down-regulated in Sp1-deficient cells are involved in cytoskeleton reorganization including multiple forms of cytokeratin.

In an additional experiment, mRNAs from $Sp1^{-/-}$19 ES cells that were supplemented with a human Sp1cDNA (rescue68 cells, protein expression shown in chapter 3) were compared with mRNA from a clone that went through the same puromycine selection procedure, but did not contain the Sp1 transgene. However, no increase in expression of identified down-

regulated genes in Sp1-deficient cells could be detected, except for calcyclin that showed a two-fold increase in the rescue68 mRNA compared to the Sp1-deficient clone.

gene	accession number	fold changes							
		11k chips				19k chips			
		exp.1	exp.2	exp.3	average	exp.1	exp.2	exp.3	average
architectural proteins									
endoA [1] hcytokeratin8	d90360		-3	-5.1	-4.1				
EndoA cytokeratin	TC27291_s_at					-4.6	-1.9	-4.6	-3.7
endoB hcytokeratin18	m11686		-2.8	-3.9	-3.4				
mcytokeratin19	m28698		-12.7	-23.2	-18				
B tropomyosin	m81086		-4.1	-3	-3.6				
Tropomyosin beta subunit, skeletal muscle						-4.2	-3.2	-2.4	-3.3
collagen type I	msa117.0		-6.3	-1.8	-4.1				
talin	msa2712.0		-2.8	-4.6	-3.7				
growth factor induced glycoprotein	X67644		-3.6	-4.5	-4.1				
annexinIII	TC28979_at					-4.1	-2.6	-3.8	-3.5
Lectin Mac-2 (=galectin3)	TC39719_f_at					-3.5	-11	-4.2	-6.2
regulatory proteins									
EST 14-3-3 σ homolog	aa670807		-16.5	-15.5	-16				
14-3-3 protein stratifin	TC23274_at					-13.6	-2.6	-4.9	-7
mouse tissue factor	m26071-2		-3.3	-5.4	-4.4				
gelsolin	j04953		-7.6	-7.6	-7.6				
calcyclin [2]	m37761		-4.2	-4.7	-4.5				
TDAG51-T cell apoptosis	U44088		-2.6	-5.2	-3.9				
Goliath protein (G1 protein) (Drosophila)	TC32281_at					-3.8	-6.4	-4.5	-4.9
TEF-1	TC33141_s_at					-3	-4.7	-3.7	-3.8

Table 1: Known genes and ESTs recognized by microarray analysis of Sp1 heterozygous versus Sp1 homozygous knock out ES cells. Only those are listed that show an average expression decrease in Sp1-deficient cells of factor3 or more. Exp.1 = experiment 1: Sp1+/-20/8 RNA compared with Sp1-/-19 RNA. Exp.2 = Sp1+/-11 RNA compared with Sp1-/-20 RNA. Exp.3 = repitition of experiment 1 with preparation of new cRNA from the same batch of mRNA.

accession number of EST	fold changes							
	11k chips				19k chips			
	exp.1	exp.2	exp.3	average	exp.1	exp.2	exp.3	average
AA420397		-5.3	-2.2	-3.8				
aa239576		-4.5	-6.6	-5.6				
aa407367		-3.7	-3.6	-3.7				
aa414922		-6.6	-6.3	-6.5				
aa591007		-3.6	-3.1	-3.4				
aa600542		-2.5	-3.9	-3.2				
TC22126_g_at					-10.6	-4.1	-7.0	-7.2
TC26378_at					-3.7	-8.2	-6.7	-6.2
TC26378_g_at					-6.3	-2.3	-6.9	-5.2
TC29206_at					-3.3	-5.9	-4.1	-4.4
TC29651_at					-3	-2.3	-3.9	-3.1
TC31277_at					-2.6	-4.2	-2.2	-3
TC31283_at					-5.2	-2.6	-2.8	-3.5
TC32093_at					-4.3	-4.3	-3.3	-4
TC33498_at					-5.5	-3.6	-4.3	-4.5
TC33957_i_at					-2.3	-15	-3.8	-7
TC33957_s_at					-6.3	-27.9	-7.5	-13.9
TC35523_at					-2.9	-4.2	-2	-3
TC37556_at					-10.1	-7.1	-6.1	-7.8
TC38819_at					-2.4	-4.6	-3.6	-3.5
TC14557_f_at						-3.4	-2.8	-3.1
TC23274_at						-2.6	-4.9	-3.8
TC27291_s_at						-1.9	-4.6	-3.3
TC28979_at						-2.6	-3.8	-3.2
TC29158_at						-3	-4.4	-3.7
TC30010_at						-4	-2.2	-3.1
TC30483_g_at						-5.7	-3.2	-4.5
TC32281_at						-6.4	-4.5	-5.5
TC32971_g_at						-4	-2.3	-3.2
TC33141_s_at						-4.7	-3.7	-4.2
TC34469_at						-3.9	-2.4	-3.2
TC35711_at						-3.6	-3.6	-3.6
TC35925_g_at						-4.5	-2.4	-3.5
TC39719_f_at						-11	-4.2	-7.6

Verification of target genes

Down-regulation of some of the known genes in Sp1-deficient cells was tested by Northern blot analysis (Figure 1 and Figure 2A) on total RNA from different ES cell lines. For comparison, also RNA from wildtype ES cells (E14), rescue68 cells and $Sp3^{-/-}$ Es cells was used. 14-3-3σ, one of the genes that showed dramatic down-regulation in the microarray analysis, shows down-regulation in $Sp1^{-/-}$ cells as well in the Northern blot analysis. However, the detected level of down-regulation is much less. When defining the 14-3-3σ RNA expression level of the wildtype E14 ES cell line as 100%, $Sp1^{+/-}$ ES cells express 1.5 times less 14-3-3σ RNA, $Sp1^{-/-}20$ cells 2.8 times less, rescue68 cells 3.5 times less and surprisingly, $Sp3^{-/-}$ cells 7 times less.

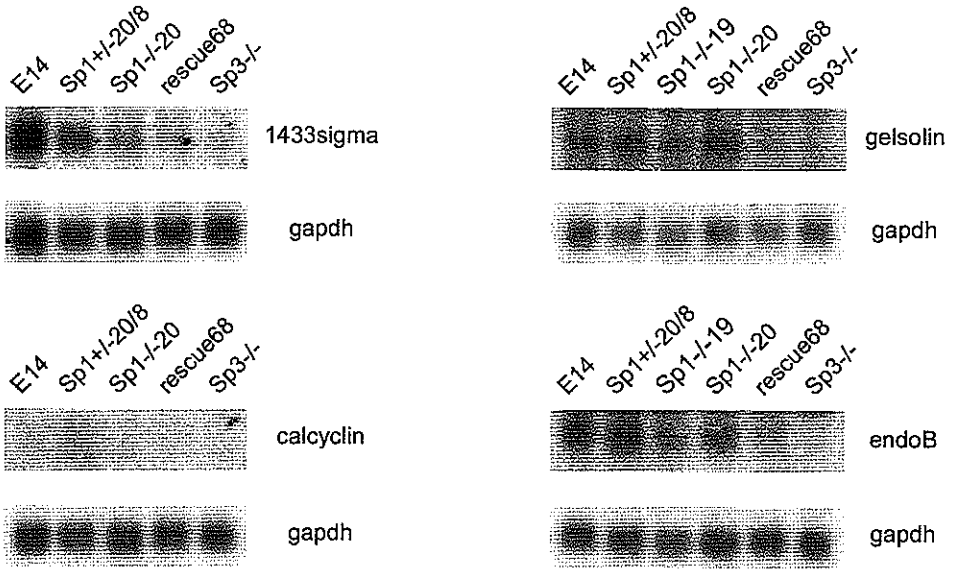

Figure 1: Northern blot analysis for verification of putative target genes in different ES cells. Blots were re-hybridised with gapdh as probe.

Gelsolin expression in Sp1-deficient ES cell lines is half of the Sp1 heterozygous ES cell line, however this expression is at the same level than in the wildtype ES cells. Expression levels in rescue68 and $Sp3^{-/-}$ cells are almost undetectable.

For endoB (the mouse homologue of human cytokeratin 18) no significant down-regulation in Sp1-deficient ES cells can be detected. Again, expression levels in rescue68 and $Sp3^{-/-}$ cells are almost undetectable.

For calcyclin, overall expression levels were very low and only very faintly detectable in Sp1 heterozygous ES cells.

For cytokeratin19 (Figure 2A) a 4-fold difference in expression level between the Sp1 heterozygous and homozygous ES cell line could be detected. Again, as for 14-3-3σ, differences in the microarray analysis were much higher (18-fold in average).

Methylation analysis of the cytokeratin19 promoter by Southern blot

As mouse cytokeratin19 is indeed down-regulated in $Sp1^{-/-}$ cells and its promoter sequence was known (Pubmed AF237661), we wanted to test whether we could detect differences in the methylation status of its promoter. The genomic organization of the murine cytokeratin19 gene is shown in Figure 2C. Within the regulatory region of the mouse cytokeratin19 gene, three Sp1 binding sites are found directly in front of the transcription start site, a CpG island can be found close to the promoter. Isolated genomic DNA from different ES cell lines was

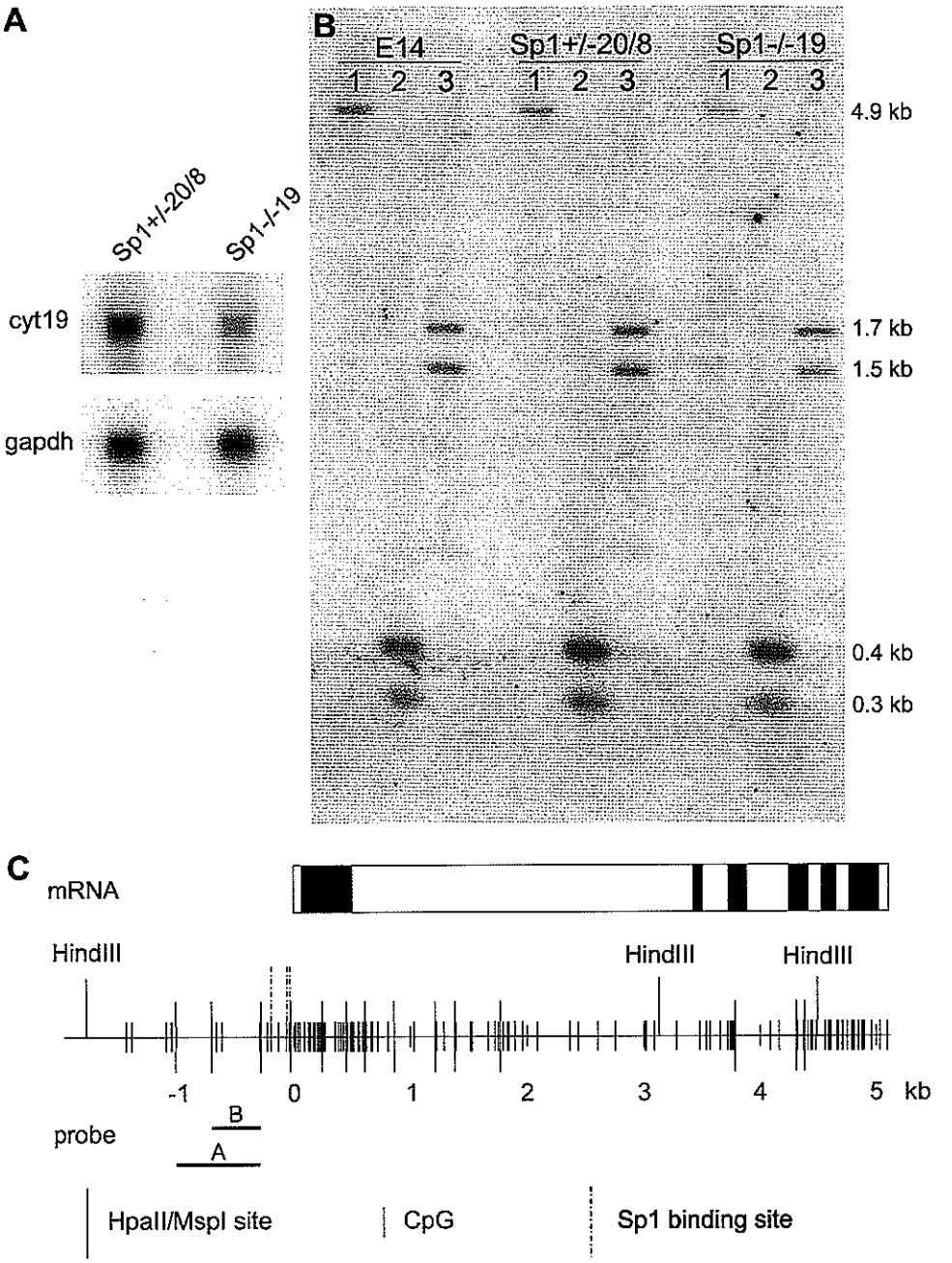

Figure 2: (A) Northern blot of cytokeratin19 expression in Sp1+/- and Sp1-/- ES cells. Quantification yields a 4fold reduction in Sp1-/- ES cells. (B) Southern blot analysis of the cytokeratin19 promoter using genomic ES cell DNA. DNA was digested with HindIII only (1) or in addition with MspI (2) or in addition with HpaII (3). Probe A, depicted in (C) was used for hybridisation. Sizes of obtained fragments are given on the right. (C) Genomic organisation of the mouse cytokeratin19 gene. Exons are represented by black boxes. HpaII/Msp1I sites, CpG dinucleotides and Sp1 binding sites are depicted.

digested with different enzymes and a Southern blot was done using probe A depicted in Figure 2C. HindIII digest yields a 4.9kb fragment as expected (Figure 2B). Additional digest with the methylation-insensitive enzyme MspI yields two fragments of 0.3 and 0.4 kb length. As probeA spans a MspI site, and is flanked by two MspI sites, these fragments represent the sequence that is covered by the probe. A combined HindIII/HpaII digest, with HpaII being a methylation-sensitive enzyme, gives rise to two bands of 1.5 and 1.7 kb. These patterns can be explained, when the first two HpaII sites are methylated, the third is partially methylated (1.5kb band) and the HpaII site directly in front of the promoter is mostly unmethylated. A Southern blot using probe B yielded the same patterns, except that the MspI digest only showed the 0.4kb band (not shown). $Sp1^{+/-}$20/8 and $Sp1^{-/-}$19 ES cells show the same patterns, although the expression level is reduced in the $Sp1^{-/-}$19 cells by factor 4. For the promoter region tested with this method, we could not detect any differences in the methylation status of the cytokeratin19 promoter.

Discussion

Detection of Sp1 target genes

DNA microarray analysis of Sp1-deficient versus Sp1 heterozygous ES cells revealed a number of genes that are potentially regulated by Sp1. Differential expression of some of these genes was verified by Northern blot analysis. Although this confirmed the qualitative differences in expression levels observed by microarray analysis, large quantitative differences were found between the two methods. For instance, DNA chip analysis of 14-3-3σ expression yielded a 7- to 16-fold difference between heterozygous and homozygous Sp1 knockout ES cells. Although a different pair of Sp1 heterozygous/homozygous cells was used for Northern blot analysis, only a twofold difference could be detected. The same was seen for cytokeratin19, showing an 18-fold difference by DNA chip analysis and only a fourfold difference by Northern blot analysis.

In addition, only the genes that showed a very high differential expression on the chips could be verified by Northern blot analysis. This is unexpected, as usually Northern blot analysis is thought to be more sensitive to minor differences than DNA chip analysis. As a cytokeratin19 Northern blot using polyA+ RNA instead of total RNA gave as well a fourfold down-regulation (not shown), it is unlikely, that these differences between the two analysis methods arise from use of either polyA+- versus total RNA.

The rescue68 ES cell line, which expresses a human Sp1-transgene in the Sp1-deficient background of mother clone $Sp1^{-/-}$19, does not show up-regulation of 14-3-3σ to the levels seen in Sp1 heterozygous cells. Probably, the amount of transgene-derived Sp1 is too low to

sustain robust expression levels of 14-3-3σ. Rescue68 and, surprisingly, $Sp3^{-/-}$ cells usually show the lowest expression levels of the genes tested. However, it has not been tested if this phenomenon is due to clonal variation, or if 'Sp1 target genes' are also 'Sp3 target genes' in ES cells.

Methylation status of the cytokeratin19 promoter in ES cells

From the three Sp1 sites at –198, -62 and –33 bp, depicted in Figure 2C, two (at –198 and –62) are also present in the human K19 gene (Lussier et al., 1990). Gut-enriched Krüppel-like factor (GKLF) and Sp1 have been shown to be important for the transcriptional regulation of K19 (Brembeck and Rustgi, 2000).

Methylation of promoter regions and CpG islands has often been described to result in transcriptional down-regulation of genes. As Sp1 has been implied in the maintenance of the methylation-free status of CpG islands (Brandeis et al., 1994; Macleod et al., 1994) we asked if we could detect methylation differences in Sp1-containing ES cells that express K19 at high levels and in Sp1-deficient ES cells that show reduced K19 RNA levels. However, Southern blot analysis did not reveal differences in the methylation status of the promoter region between the two ES cell lines.

Possible significance of found Sp1 target genes

Expression of 14-3-3σ was found to be highly reduced in Sp1-deficient ES cells in all microarray analyses. The reduced expression could be verified by Northern blot, although to a lesser extent. 14-3-3σ has a major impact on the G2/M cell cycle checkpoint after DNA damage through the regulation of the cellular distribution of the Cdc2 kinase (Chan et al., 1999; Hermeking et al., 1997). A more detailed analysis of 14-3-3σ expression and its function in ES cells can be found in chapter 3.

One striking point that emerged from the DNA microarray analysis is that a number of differentially expressed genes play a role in the cytoskeleton and its regulation. Three cytokeratins appear on the microarray list: the mouse equivalents of cytokeratin8 K8 (endoA), cytokeratin18 K18 (endoB) and cytokeratin19 K19. Cytokeratins are the most diverse group among the intermediate filaments and represent the most abundant proteins in epithelial cells (Fuchs and Weber, 1994). Their main function is the protection of epithelial cells from mechanical stress. Obligatory heterodimeric subunits of acidic type I (keratins K9-K20) and basic type II (keratins K1-K8) assemble to build a cytoplasmic, highly dynamic network. Different sets of pairs of type I and type II keratins are differentially expressed in most epithelial cells at various stages of development and differentiation (Fuchs and Green, 1980; Moll et al., 1982; Wu et al., 1982).

The phenotype of K8 knockout mice is strongly dependent on the genetic background of the mouse strain. In C57Bl/6 background, 94% of all K8 homozygous KO mice died around E12.5, showing growth retardation, internal bleeding, with an abnormal accumulation of erythrocytes in the fetal liver (Baribault et al., 1994; Baribault et al., 1993). However, in the FVB/N background the proportion of viable homozygotes increased with 50%. Surviving animals developed colorectal hyperplasia and inflammation as adults, the females showed a reduced reproductive capacity (Baribault et al., 1994).

K18 KO mice are viable, fertile and have a normal life span. However, old K18 KO mice develop a liver pathology with abnormal hepatocytes containing K8-positive aggregates (Magin et al., 1998). K19 KO mice are as well viable, fertile and appear normal (Tamai et al., 2000).

In order to address the question of functional redundancy during development, keratin compound knock-out mice were generated. The K8/K19 (Tamai et al., 2000) as well as the K18/19 (Hesse et al., 2000) compound KO mice die in utero around E10 or E9.5, respectively. Both compounds showed defective placental development, resulting in flooding of the maternal blood into the embryonic placenta.

So far, only cytokeratin19 could be shown to be significantly down-regulated in Sp1-deficient ES cells by Northern blot analysis. However, it would be interesting if Sp1 plays a role in the regulation of these genes during development. It could be, that Sp1-deficiency results in the de-regulation of the cytokeratin network which could contribute to the embryonically lethal phenotype of Sp1-deficiency in mice (Marin et al., 1997). To study the impact of Sp1 on cytokeratin expression during mouse development, the use of a conditional Sp1 knockout (as described in Chapter 4) would be useful.

Materials and Methods

Isolation of total RNA and preparation of polyA$^+$-RNA

Total RNA was prepared from exponentially growing ES cell cultures with TRI REAGENT (Sigma) according to the manufacturer's instructions. Briefly, after removal of the ES medium, cells were washed once with PBS. After the cells were lysed for 5 minutes at room temperature in TRI REAGENT, a chloroform extraction was done. Samples were left for 10 minutes at room temperature, before being centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous phase containing the RNA was precipitated with isopropanol for 10 minutes at room temperature and centrifuged at 12000 rpm for 15 minutes at 4°C. RNA pellets were washed twice with 75% ethanol and stored therein at –80°C until usage.

Before the preparation of mRNA, total RNA samples were pooled and dissolved in DEPC-treated water. RNA-concentrations were determined by OD measurement. The quality of the RNA was checked on a non-denaturing 1% agarose gel for a 2:1 ratio of 28S:18S RNA.

PolyA$^+$-RNA was prepared using Oligotex mRNA Midi Kit (Qiagen) according to the manufacturer's instructions. In order to remove ribosomal RNAs completely from the RNA preparations, the oligodT selection was done twice. Therefore the RNA samples after the first selection round were pooled and used after determination of the concentration in a second round. mRNA samples were dissolved in DEPC-treated water and pooled. Again the quality was determined on a non-denaturing agarose-gel for the disappearance of the 18S and 28S rRNA bands as well as for the isolated polyA$^+$-RNA showing a population of RNA molecules ranging from high-molecular to low-molecular species. mRNA samples were stored at –80°C until further usage.

Microarray analysis

2 µg of mRNA was reversed transcribed using $T7(dT)_{24}$ and labelled with biotinylated ribonucleotides incorporated by T7 RNA polymerase. Resultant RNA was fragmented and hybridised to an oligonucleotide microarray representing 11000 (11k) and 19000 (19k) gene elements according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). mRNA samples from two independent Sp1-/- ES clones were compared with two independent Sp1+/- ES clones.

Northern Blot analyses

10 µg of total RNA was separated on 1% agarose gel containing 0.02 M MOPS [3-(N-morpholino)propanesulfonic acid], 5 mM sodium acetate, 1mM EDTA and 2.2M formaldhyde and blotted on to nylone membranes and hybridised under standard conditions (Sambrook). Gene-specific probes were generated as follows:

14-3-3 sigma:

226 bp fragment generated by PCR amplification from E14 wildtype cDNA using the sense primer (5'-GCCTTTCCCAAACCCTGAAT-3') and the antisense primer (5'-ACCTCCTGGATTATTCGGAT-3').

Gelsolin:

331 bp PCR fragment using the sense primer (5'-CTAAGCGGTACATCGAGACA-3') and the antisense primer (5'- AGACCCTGAGTTTCCACAAG-3').

Calcyclin:

304 bp PCR fragment using the sense primer (5'- CCAGTGATCAGTCATGGCAT-3') and the antisense primer (5'-AGTCATCTCAACGGTCCCAT-3')

EndoB:

271 bp PCR fragment using the sense primer (5'-AACTGGACAAGTACTGGTCT-3') and the antisense primer (5'-CCCTCTGCCCGAGTTTGTGC-3')

Cytokeratin19:

611 bp PCR-fragment using the sense primer (5'-GCTGACTCTGGCCAGGACTG-3') and the antisense primer (5'-TGGGCAGATTGTTGTAGTGG-3').

All PCR reactions were done for 30 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min).

The Gapdh probe was a gift from R. Ferreira (Rotterdam).

Signal intensities were quantified using ImageQuant program (Molecular dynamics) and were normalized against Gapdh signal intensities.

Methylation analysis using Southern blotting

About 150-200 µg of genomic ES cell DNA was digested overnight at 37°C with the first restriction enzyme (HindIII). The restricted DNA was once extracted with phenol/chloroform before being precipitated with 1/10 volume 2M sodium acetate pH5.6/ethanol at –80°C. DNA pellets were washed once with 70% ethanol and dissolved in water at 4°C. DNA concentrations were measured again and for the second digest with methylation-sensitive or –insensitive restriction enzymes 15µg of this pre-restricted DNA was used. Subsequently standard southern blotting was performed. The Southern probes for cytokeratin19 were generated by PCR. Probe A was generated by use of the sense primer (5´-AAAGATCCAGAAAGATCTGG-3´) and the antisense primer (5´-GGAGTGACGATATCACTCTG-3´) generating a 710 bp probe depicted in Figure 2.

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Chapter 3

Effects of Sp1 protein levels in ES cells on sensitivity to ionising radiation and on their ability to contribute to chimeric embryos

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Abstract

Sp1-deficiency results in mid-gestational embryonic lethality in mice, whereas Sp1-deficient murine embryonic stem cells are viable. $Sp1^{-/-}$ ES cells grow normally under standard cell culture conditions, however are incapable of contributing to chimeric embryos after embryonic day 10 (Marin et al., 1997). Here we report that Sp1-deficient ES cells are hypersensitive to ionising radiation. Expression of a human Sp1 cDNA in $Sp1^{-/-}$ ES cells only partially rescued the radiation hypersensitivity, but enables these cells to efficiently contribute to chimeric embryos. 14-3-3σ, a protein described as major G2/M checkpoint control factor, has been detected as a target gene of Sp1 in a DNA chip analysis. Down-regulation of 14-3-3σ RNA and protein in $Sp1^{-/-}$ ES cells is demonstrated, forced expression of a 14-3-3σ transgene however could not rescue the hypersensitive phenotype of Sp1-deficient ES cells to ionising radiation. No gross abnormalities in the cell cycle of $Sp1^{-/-}$ ES cells compared to wildtype ES cells could be detected. Therefore, although 14-3-3σ appears to be a genuine target gene of Sp1, its down-regulation is not the only cause of the radiation hypersensitivity of $Sp1^{-/-}$ ES cells.

Introduction

Sp1 belongs to a transcription factor family that is characterised by a DNA binding domain consisting of three zinc fingers from the Cys_2-His_2-type (for review see (Bouwman and Philipsen, 2002; Philipsen and Suske, 1999). Sp1 is expressed ubiquitously in the mouse, although expression levels vary between different tissues and during differentiation (Saffer et al., 1991). Sp1 knockout mice die *in utero* around embryonic day 10 (Marin et al., 1997). Although the formation of differentiated structures like heart, blood and limbs occurs, the embryos are severely retarded. In contrast, ES cells with both Sp1 alleles functionally inactivated grow normally under standard culture conditions. As Sp1 has been implicated in the regulation of many genes, its dispensability in ES cells has been unexpected.

The cellular defect of Sp1-deficiency is cell-autonomous, as chimeric embryos show high contributions of Sp1-deficient ES cells to all tissues during early embryonic development, whereas their contribution to the embryo declines rapidly around E10. In newborn animals, Sp1-deficient cells are not detectable in any tissue (Marin et al., 1997).

The ability of cells to sense and repair spontaneous and induced DNA double-strand breaks (DSBs) is essential for their survival and the maintenance of genomic integrity. In eukaryotes, DSBs are mainly repaired by the two major pathways of non-homologous end-joining (NHEJ) or homologous recombination (HR) (reviewed in (Cromie et al., 2001; Hoeijmakers, 2001)).

14-3-3 proteins are present in all eukaryotic organisms. The 14-3-3 family comprises seven family members in mammals. The small (about 30 kDa) acidic proteins do not possess a detectable catalytic function, but are phosphoserine/phosphothreonine binding modules (for review see (Muslin and Xing, 2000; Tzivion and Avruch, 2002; van Hemert et al., 2001; Yaffe, 2002)). Roles in important cellular processes have been ascribed to this protein family, like in signal transduction, cell cycle control, apoptosis, stress response and malignant cell transformation. By binding to their phosphorylated target protein, 14-3-3 proteins can influence protein-protein interactions by acting as adaptor proteins, regulate the subcellular localisation of proteins or the activity of enzymes. More than 100 different binding partners of 14-3-3 proteins have been described.

The 14-3-3σ isoform (also called stratifin or HME1 for human mammary epithelium) has been originally discovered through its expression in differentiating epithelial cells (Leffers et al., 1993; Prasad et al., 1992). It is strongly up-regulated in human colorectal cancer cells upon exposure to ionising radiation or DNA damaging agents (Hermeking et al., 1997). The DNA-damage induced upregulation of 14-3-3σ in human colon carcinoma cells has been shown to occur through p53 mediated increase in gene transcription (Hermeking et al., 1997).

BRCA1-/- mouse ES cells show reduced 14-3-3σ RNA levels and exit G2/M arrest prematurely after ionising radiation when compared to wildtype cells (Aprelikova et al., 2001). Human colorectal cancer cells lacking 14-3-3σ initiate a G2/M arrest following DNA damage but are unable to maintain cell cycle arrest and die due to mitotic catastrophe (Chan et al., 1999). It has been proposed that the failure of 14-3-3σ-deficient cells to sustain G2/M arrest is due to their inability to sequester cyclinB-Cdc2 in the cytoplasm to give the cells time to repair damaged DNA (Chan et al., 1999; Laronga et al., 2000). Clinical observations suggest that 14-3-3σ could function as a tumour suppressor. It is frequently down-regulated in breast and gastric cancer and human hepatocellular carcinoma (Iwata et al., 2000; Prasad et al., 1992; Vercoutter-Edouart et al., 2001) due to hypermethylation of the 14-3-3σ promoter (Ferguson et al., 2000; Iwata et al., 2000; Umbricht et al., 2001).

Here, we describe the new finding that Sp1-/- ES cells are hypersensitive to ionising radiation and that 14-3-3σ is a target gene of Sp1 in ES cells. However, complementation studies show that down-regulation of 14-3-3σ is not solely responsible for this hypersensitive phenotype of Sp1-deficient ES cells.

Sp1-deficient ES cells are hypersensitive to ionising radiation (IR)

In order to analyse a possible role of Sp1 in DNA double strand break (DSB) repair we performed colony formation assays to assess the IR sensitivity of Sp1-deficient ES cells compared to normal ES cells. Two independent $Sp1^{-/-}$ ES cell clones were highly sensitive to IR as compared with the wildtype E14 ES cells, showing a comparable sensitivity to that observed with $Rad54^{-/-}$ cells (Figure 1). RAD54 is a member of the SWI/SNF family of DNA-dependent ATPases, its disruption in ES cells results in hypersensitivity to ionising radiation (Essers et al., 1997). Earlier studies showed that Sp1-deficient ES cells are not hypersensitive to UV radiation (M. Marin, unpublished results). Therefore, Sp1 contributes to the survival of murine ES cells upon the cellular insult of DNA DSBs induced by ionising radiation.

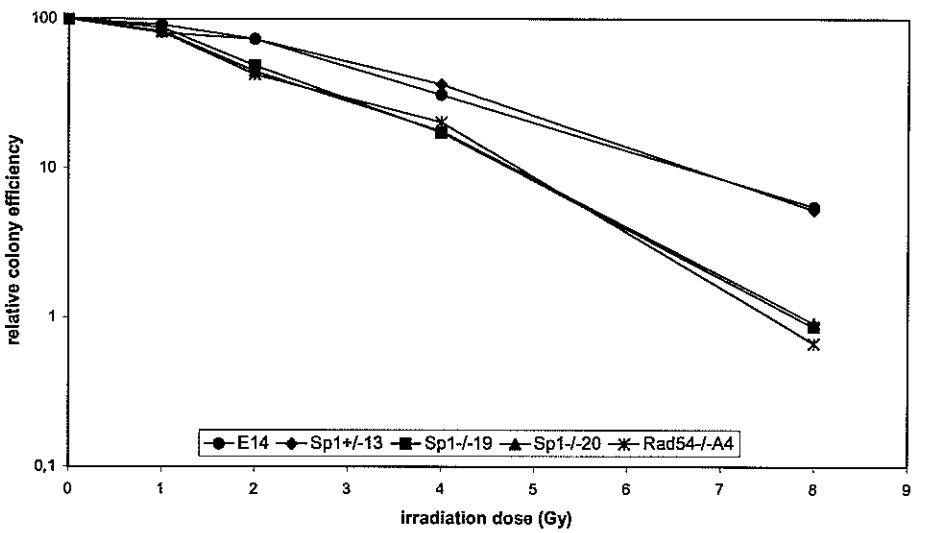


Figure 1: Effects of ionising irradiation on the survival of ES cells.
The survival of ES cells is measured as their colony-forming ability after irradiation, which is normalised against the non-irradiated values. This relative colony efficiency is blotted as a function of the irradiation dose given in gray (Gy). All measurements were performed in triplicate.

Sp1 cDNA expression restores the cell-autonomous defect of Sp1-deficient ES cells in embryonic chimeras

In order to see, if forced expression of Sp1 can restore the cellular deficiencies of $Sp1^{-/-}$ ES cells, human Sp1 cDNA was cloned into an expression vector for its expression in ES cells under the control of the phosphoglycerate kinase (PGK) promoter (Figure 2A). $Sp1^{-/-}$19 ES cells were co-electroporated with the linearized Sp1 cDNA vector and a vector carrying a

puromycine resistance. Puromycine resistant clones were checked by Southern blot analysis for Sp1 transgene integration using a probe that spans the zinc finger region, which is deleted from the Sp1 gene in $Sp1^{-/-}$ ES cells (not shown). Sp1 transgene expression was analysed by Western blotting of nuclear protein extracts. Bandshift analysis with these protein extracts and an oligonucleotide carrying a Sp1 binding site showed, that the Sp1 protein translated from the cDNA transgene was able to bind to its DNA recognition sequence *in vitro* (not shown). The expression analysis of four clones is depicted in Figure 2B. As loading control, the same western blot was re-probed with an antibody against TFIIH subunit p62. For comparison, nuclear extracts of wildtype E14, heterozygous and homozygous Sp1 KO cells are also shown. As the knockout cells still express the N-terminal part of the protein, this truncated Sp1 protein (KO) is visible below the full-length protein (wt) (Marin et al., 1997). Estimated from the comparison of the signal intensities, the ES clone rescue68, which shows the highest expression level of human Sp1 found in the analysed ES cells, expresses about one third to one half of the endogenous Sp1 level of the heterozygous Sp1 ES cells.

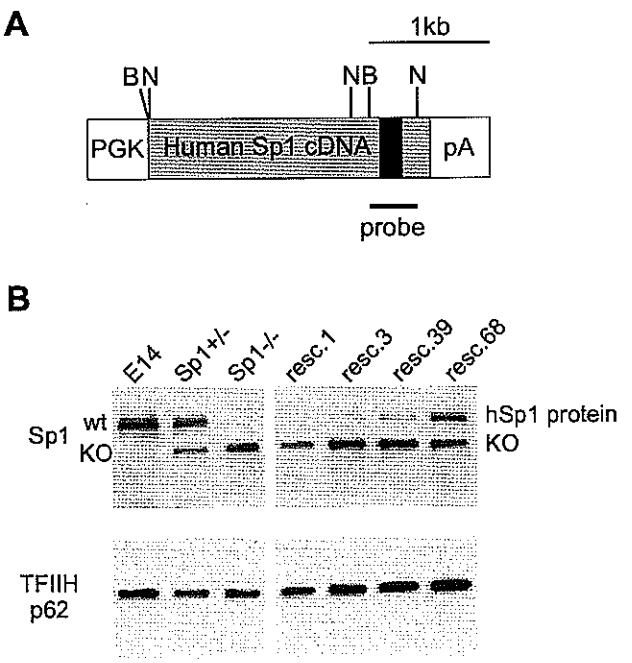


Figure 2: Expression of a human Sp1 cDNA construct in Sp1-/- ES cells.
(A) Sp1 cDNA construct. Human Sp1 cDNA was cloned into a plasmid containing the phosphoglycerate kinase (PGK) promoter and a polyadenylation (pA) signal. The zinc-finger region is depicted as black box. N, NcoI, B, BamHI.
(B) Western blot analysis on nuclear extracts of puromycine resistant Sp1-/- ES clones for Sp1cDNA expression. As control wildtype E14, Sp1 heterozygous ES cells and the Sp1-/- mother clone were used. Deletion of the zinc finger domain of Sp1 results in the expression of the N-terminal part of Sp1 (KO). The blot was re-hybridised with a TFIIH-specific antibody as loading control.

Rescue68 ES cells were injected into wildtype C57/Bl6 host blastocysts to see if they, as opposed to Sp1-deficient ES cells, can contribute to normal mouse development. At embryonic day 9.5, embryos were isolated and stained for lacZ expression. As $Sp1^{+/-}$ and

$Sp1^{-/-}$ ES cells express an IRES-lacZ fused to the remaining N-terminal part of Sp1, lacZ expression can be used to detect $Sp1^{-/-}$ ES cells. As shown in Figure 3, embryos that are

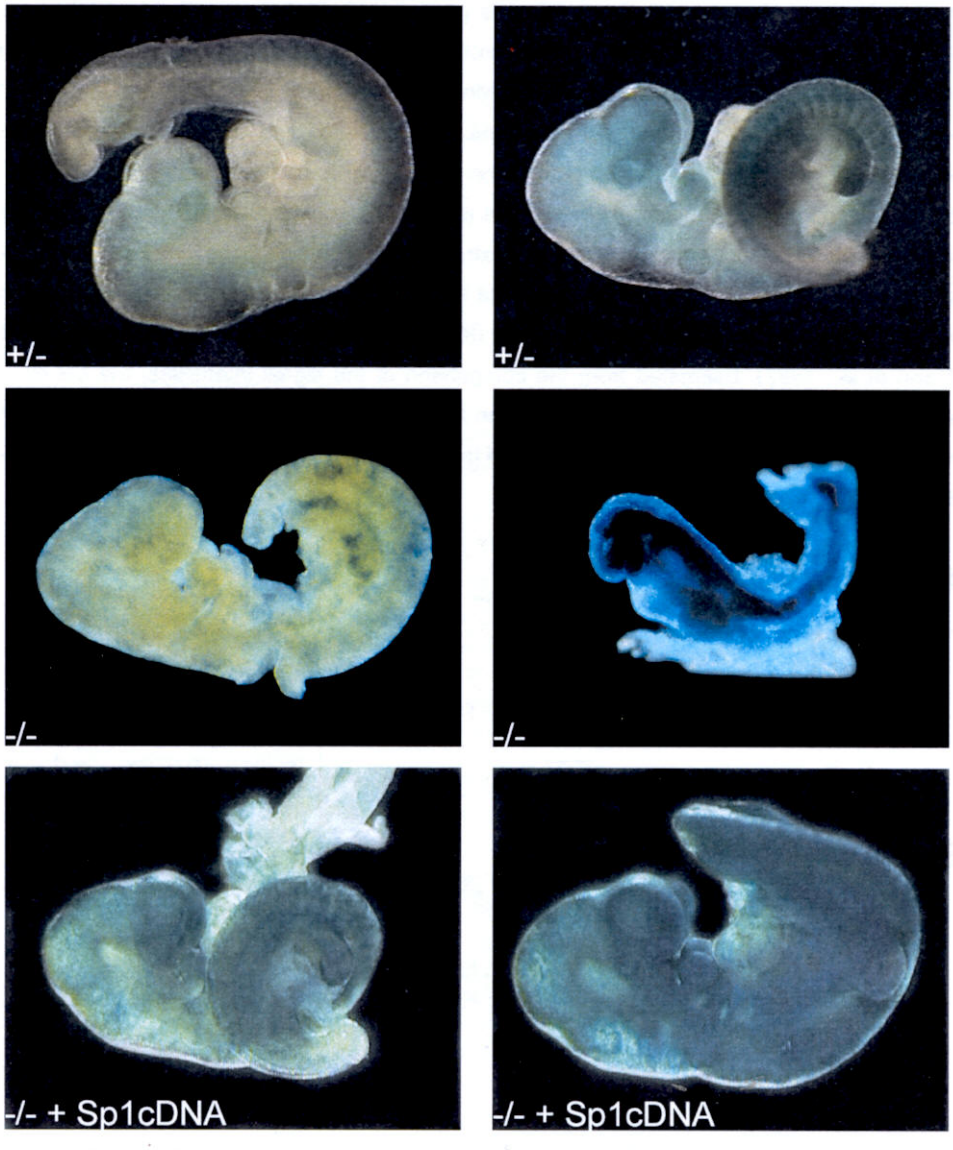


Figure 3: Contribution of Sp1+/-, Sp1-/- and rescue68 (Sp1-/- + Sp1 cDNA) ES cells to chimeric embryos at E9.5. ES cells of the given genotypes were injected into host C57Bl/6 blastocysts. Embryos were isolated at embryonic day 9.5, stained for lacZ expression and photographed. Pictures of the upper and middle panel originate from (Marin, 1997).

highly chimeric with $Sp1^{-/-}$ cells (middle panel) are severely retarded at E9.5, compared to chimeric embryos resulting from injection of Sp1 heterozygous ES cells (upper panel). However, embryos resulting from injection of rescue68 ES cells appear normal even when

highly chimeric (lower panel). Embryos of two pregnant foster mice were allowed to come to term and two normal appearing male chimeras were obtained, one of them being highly chimeric as judged by fur colour. These male chimeras were fertile, but did not give germline transmission.

Sp1 cDNA expression in Sp1 KO ES cells partially rescues hypersensitivity to ionising radiation

Rescue68 ES cells were subjected to the colony formation assay. As can be seen in Figure 6, rescue68 cells show ionizing radiation sensitivity comparable to wildtype or Sp1 heterozygous ES cells at lower irradiation doses. However, at the high dose of 8 Gy the sensitivity restoration cannot be maintained.

Effects of expression of Sp1 target gene 14-3-3σ on hypersensitivity to ionising radiation

In a screen for Sp1 target genes using RNA from Sp1 heterozygous and homozygous ES cells, 14-3-3σ had been discovered as a potential Sp1 target gene in ES cells. Two independent Sp1 knockout ES cell lines showed down-regulation of 14-3-3σ when compared to Sp1 heterozygous cells (see Chapter 2). As 14-3-3σ has been described as key regulator of a G2/M cell cycle arrest upon irradiation (Chan et al., 1999), we were interested in this gene, as its down-regulation in Sp1 knockout ES cells might contribute to their hypersensitivity against ionising radiation.

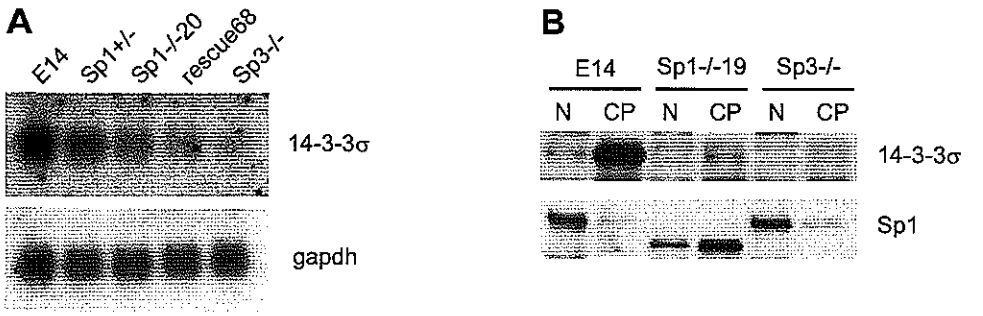
The Northern blot in Figure 4A shows, that 14-3-3σ is indeed down-regulated in $Sp1^{-/-}$ ES cells. By quantification of the signal intensities and their normalisation against the signal intensities for gapdh, factors of down-regulation were calculated. Defining the 14-3-3σ RNA expression level of the wildtype E14 ES cell line as 100%, $Sp1^{+/-}$ ES cells express 1.5 times less 14-3-3σ RNA, $Sp1^{-/-}$20 cells 2.8 times less, rescue68 cells 3.5 times less and $Sp3^{-/-}$ cells 7 times less.

The forced expression of human Sp1 protein in $Sp1^{-/-}$ ES cells (rescue68) has no effects on 14-3-3σ transcript levels. This is consistent with the observation of the DNA chip analysis that was performed with two ES clones that were transgenic for the Sp1 cDNA transgene. One of those did not express detectable levels of human Sp1 whereas rescue68 expresses about one third to one half of Sp1 heterozygous ES cells (see above).

Surprisingly, also $Sp3^{-/-}$ cells show only very low 14-3-3σ expression. The northern blot results for $Sp1^{-/-}$ and $Sp3^{-/-}$ ES cells are confirmed on the protein level by western blotting (Figure 4B). The 14-3-3σ protein is almost exclusively detectable in the cytoplasmic fraction of the ES cell extracts.

In order to be able to express 14-3-3σ as transgene in $Sp1^{-/-}$ ES cells we cloned the 14-3-3σ cDNA by PCR amplification from wildtype E14 ES cell cDNA. The obtained cDNA sequence

and its translation product are depicted in Figure 4C and correspond to Ensembl Transcript ID ENSMUST 00000041945 given in the MGSC Mouse Genome Browser. This cDNA and a



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1       ATGGAGAGAGCCAGTCTGATCCAGAAGGCCAAGTTGGCTGAACAGGCCGAACGGTATGAA
1        M  E  R  A  S  L  I  Q  K  A  K  L  A  E  Q  A  E  R  Y  E
61      GACATGGCAGCTTTCATGAAGAGCGCTGTGGAAAAGGGCGAGGAGCTCTCCTGCGAGGAG
21       D  M  A  A  F  M  K  S  A  V  E  K  G  E  E  L  S  C  E  E
121     CGAAACCTGCTTTCCGTAGCCTACAAGAACGTGGTGGGCGGCCAGAGAGCGGCCTGGAGG
41       R  N  L  L  S  V  A  Y  K  N  V  V  G  G  Q  R  A  A  W  R
181     GTCCTGTCCAGCATCGAGCAGAAGAGCAACGAGGAGGGGTCAGAAGAGAAGGGCCCCGAG
61       V  L  S  S  I  E  Q  K  S  N  E  E  G  S  E  E  K  G  P  E
241     GTGAAAGAGTACCGGGAGAAGGTAGAGACCGAGCTCAGAGGTGTGTGCGACACTGTGCTC
81       V  K  E  Y  R  E  K  V  E  T  E  L  R  G  V  C  D  T  V  L
301     GGCCTGCTGGACTCGCACCTCATCAAAGGGGCTGGAGATGCAGAGAGCCGCGTCTTCTAC
101      G  L  L  D  S  H  L  I  K  G  A  G  D  A  E  S  R  V  F  Y
361     CTGAAGATGAAGGGTGACTACTACCGCTACCTAGCCGAGGTGGCCACTGGCGATGACAAG
121      L  K  M  K  G  D  Y  Y  R  Y  L  A  E  V  A  T  G  D  D  K
421     AAGCGCATCATCGATTCTGCCCGGTCAGCCTACCAGGAGGCCATGGACATCAGCAAGAAG
141      K  R  I  I  D  S  A  R  S  A  Y  Q  E  A  M  D  I  S  K  K
481     GAGATGCCGCCTACCAACCCCATCCGCCTGGGCCTGGCCCTGAACTTTTCAGTCTTCCAC
161      E  M  P  P  T  N  P  I  R  L  G  L  A  L  N  F  S  V  F  H
541     TACGAGATAGCCAACAGCCCCGAGGAGGCCATCTCGCTGGCCAAGACCACCTTCGACGAG
181      Y  E  I  A  N  S  P  E  E  A  I  S  L  A  K  T  T  F  D  E
601     GCCATGGCCGACCTGCACACCCTCAGTGAGGACTCCTACAAGGACAGCACCCTCATCATG
201      A  M  A  D  L  H  T  L  S  E  D  S  Y  K  D  S  T  L  I  M
661     CAGCTCCTGAGAGACAACCTGACGCTGTGGACAGCCGACAGTGCTGGGGAAGAGGGTGGT
221      Q  L  L  R  D  N  L  T  L  W  T  A  D  S  A  G  E  E  G  G
721     GAGGCTCCGGAGGAGCCCCAGAGCTGA
241      E  A  P  E  E  P  Q  S  *
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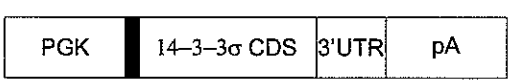

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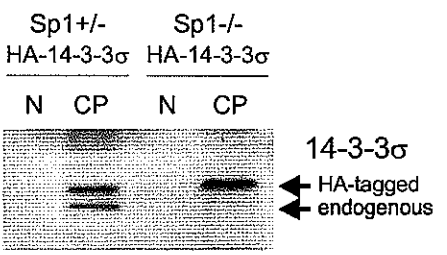


Figure 4: Sp1 target gene 14-3-3σ. (A) Northern blot analysis of RNAs from different ES cells lines using a 14-3-3σ specific probe. As loading control the Northern blot was re-probed with a gapdh-specific probe. (B) Western blot analysis of 14-3-3σ protein in nuclear (N) and cytoplasmic (CP) protein extracts from different ES cell lines. The Western blot was re-probed with a Sp1-specific antibody. (C) Nucleotide and derived amino acid sequence of the 14-3-3σ cDNA cloned from wildtype ES cell cDNA by PCR. The amino acid stretch that is specific for the σ-isoform within the 14-3-3 family (Chan, 1999) is highlighted in grey. (D) Construct for transgenic expression 14-3-3σ cDNA under the control of the PGK promoter. CDS, coding region, 3'UTR, 3' untranslated region, pA, polyadenylation signal. The HA-tag is depicted as black box. (E) Western blot analysis for the expression of the HA-tagged 14-3-3σ transgene in Sp1 heterozygous and Sp1 homozygous knock out ES cells.

part of its 3´-untranslated region were cloned into an expression vector that uses the PGK promoter to drive 14-3-3σ cDNA transcription (Figure 4D). In order to distinguish between the endogenous 14-3-3σ protein and the transgene, a haemaglutinine tag was cloned 5' to the 14-3-3σ cDNA. One Sp1 heterozygous ES cell line as well as two Sp1 homozygous KO ES cell lines ($Sp1^{-/-}$19 and $Sp1^{-/-}$20) were electroporated with this construct, along with a puromycine resistance vector. 14-3-3σ transgene integration and expression in puromycine-resistant clones were checked by PCR on genomic DNA and by western blot analysis using an antibody against the haemaglutinine tag (not shown). Figure 4E shows a western blot probed with the 14-3-3σ antibody on nuclear and cytoplasmic fractions of Sp1 heterozygous and homozygous ES cells transfected with the transgene. The endogenous 14-3-3σ is only visible in the cytoplasm of the Sp1 heterozygous ES cell line, whereas the HA-tagged 14-3-3σ transgene is seen in the cytoplasm of both ES cell lines.

In order to analyse the expression of endogenous as well as transgenic14-3-3σ protein upon irradiation, several ES cell lines were irradiated with 8 Gy. Nuclear and cytoplasmic protein extracts were prepared at certain time points after irradiation. Sp1 protein levels were analysed for the wildtype E14 ES cell line (Figure 5A). Sp1 protein is readily detectable in all nuclear protein samples, showing no dramatic expression changes upon irradiation. Extracts were also subjected to western blot analysis using a 14-3-3σ-specific antibody (Figure 5B). As can be seen for all ES cell lines, the endogenous 14-3-3σ protein is mainly detected in the cytoplasmic fraction. Visible nuclear 14-3-3σ bands might originate from contamination of nuclear fractions with rests of cytoplasmic fractions. The extracts of the $Sp1^{-/-}$19 cell line show dramatically reduced 14-3-3σ-protein levels at all time points and only very faint bands in the cytoplasmic fraction at 12, 24 and 48 hours after irradiation. In contrast to the northern result shown in Figure 4A, where the rescue68 cells do not show a transcriptional up-regulation of 14-3-3σ RNA when compared to the $Sp1^{-/-}$ cells, the rescue68 cell line shows robust 14-3-3σ protein levels.

A Sp1$^{-/-}$19 cell line that expresses transgenic HA-tagged 14-3-3σ protein shows high expression of the transgene seen as the upper band, whereas only weak expression of the endogenous protein (lower band) is detected.

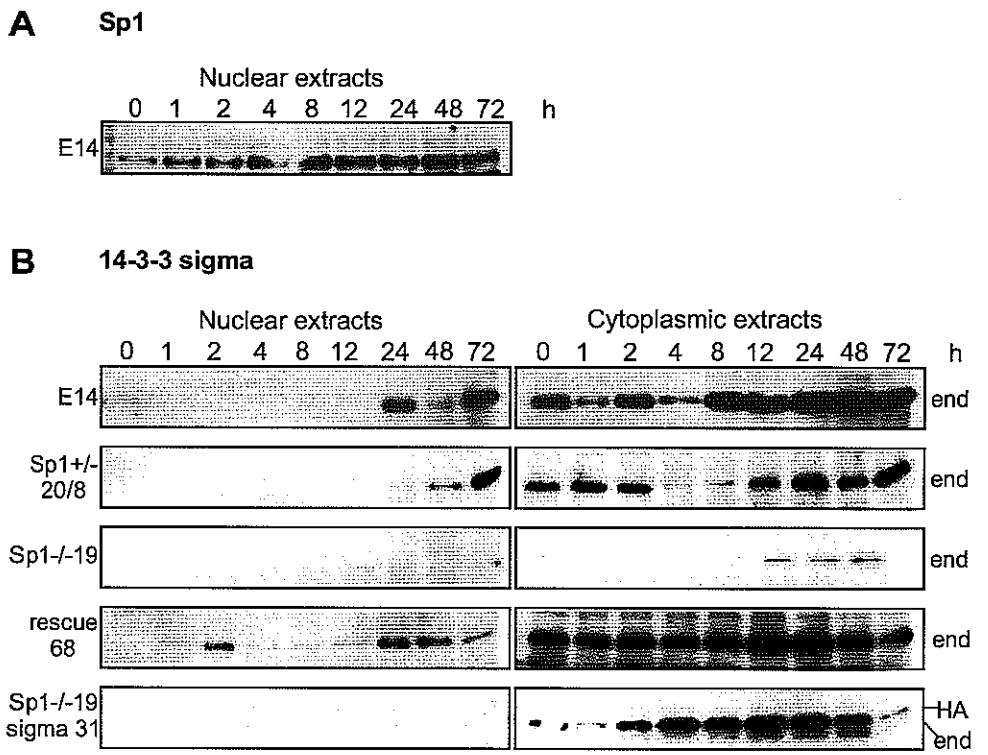


Figure 5: Western blot analysis on nuclear and cytoplasmic protein extracts prepared after given time-points (in hours) after irradiation of different ES cell lines with a dose of 8 Gy. (A) Sp1 Western blot, (B) 14-3-3σ western blots and (C) cdc2 western blots. End, endogenous 14-3-3σ, HA, HA-tagged 14-3-3σ protein.

As 14-3-3σ has been proposed to exercise control over the G2/M cell cycle checkpoint by regulating the cellular compartimentalisation of the G2/M protein kinase cdc2 (Chan et al.,

1999), we checked cdc2 distribution between nuclear and cytoplasmic fractions. As can be seen in Figure 5C, despite almost undetectable 14-3-3σ expression, $Sp1^{-/-}$ ES cells show similar distributions of cdc2 protein compared to the Sp1 heterozygous and to the rescue68 ES cell lines.

Forced 14-3-3σ expression in Sp1-/- ES cells does not restore the radiation hypersensitivity

Cell lines with different 14-3-3σ-status were subjected to a colony assay in order to analyse their survival rate upon irradiation (Figure 6). Compared to the Sp1 heterozygous ES cell line and its descendant expressing the 14-3-3σ transgene, both analysed $Sp1^{-/-}$ ES cell lines and their 14-3-3σ descendants are hypersensitive to irradiation. Again $Rad54^{-/-}$ ES cells were used as control.

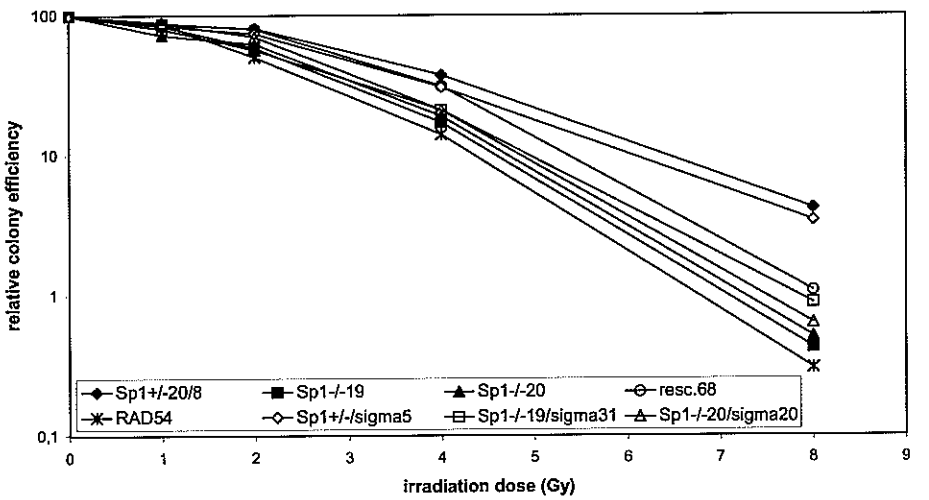
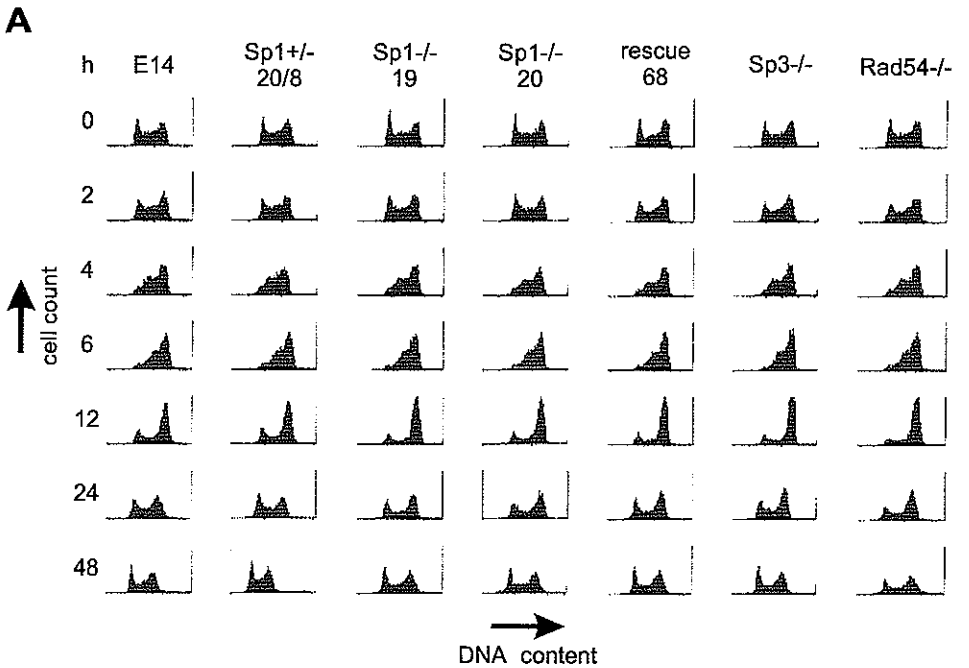


Figure 6: Colony assay of Sp1 transgenic (rescue68) and 14-3-3σ-transgenic ES clones as compared to Sp1+/- and Sp1-/- ES cells. The relative colony efficiency is blotted as a function of irradiation does in gray (Gy).

Sp1-/- ES cells show similar cell cycle kinetics upon irradiation compared to wildtype cells

Cell cycles of different ES cell lines were analysed at different time points after irradiation with 8Gy (Figure 7). As can be seen at the histograms in Figure 7A, all tested ES cell lines show in principle the same behaviour upon irradiation. Before irradiation, the histograms show a left peak, which represents cells in G0/G1 and is separated from the right peak representing cells in G2/M phase by a plateau depicting cells in S-phase. Upon irradiation this picture changes dramatically. The G0/G1 peak decreases and is virtually disappeared at 4 hours after irradiation. Meanwhile the portion of cells in S-phase increases slightly, before

decreasing to very low levels at 12 hours after irradiation. The portion of cells in G2/M-phase continuously increases with its peak at the 12-hour time-point, after which it decreases again.



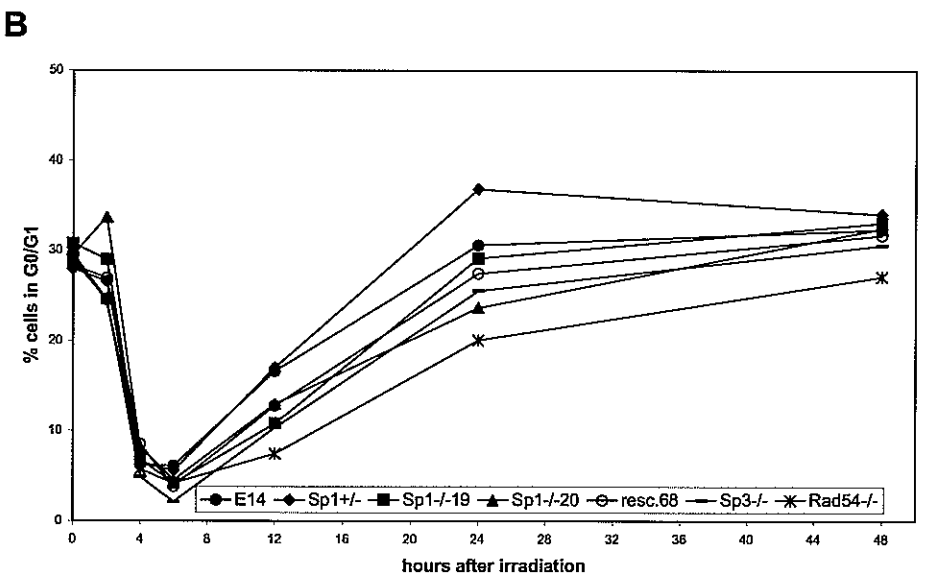
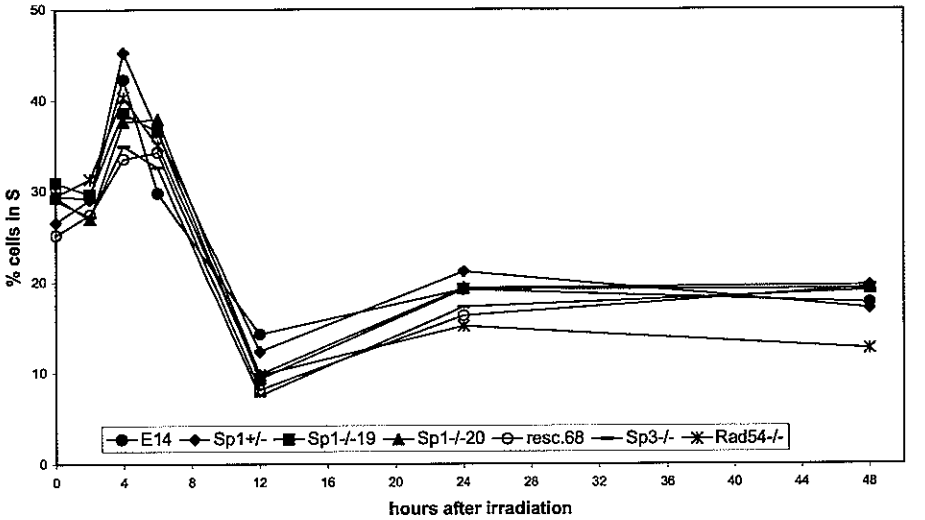


Figure 7: Cell cycle analysis of different ES cell lines after irradiation (8Gy). (A) FACS analysis showing histograms of ES cells stained with propidiume iodide at certain time points after irradiation. Percentages of ES cells in G0/G1-phase (B), S-phase (C) and G2/M phase (D) are depicted as function of time after irradiation.

C



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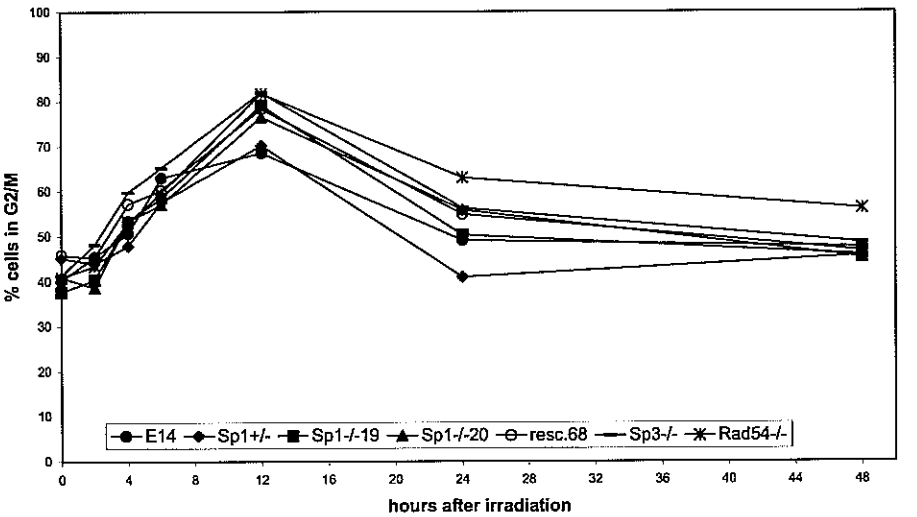

At 48 hours after irradiation, the picture of unirradiated ES cells is almost restored, however less cells are in S-phase than before irradiation. In Figure 7B, C and D the percentages of ES cell lines in G0/G1-, S- and G2/M-phase, respectively, are depicted as a function of time after irradiation. The kinetics of the cell cycle response to irradiation, as described above for the different ES cell lines is very similar.

However, a trend is visible. From 12 hours after irradiation onwards, there are less cells in G0/G1 within the RAD54$^{-/-}$ ES cell population, and more cells in G2/M-phase than in the

wildtype ES cell population. The values for Sp1 KO-, rescue68 and $Sp3^{-/-}$ ES cells are found in between.

Discussion

As Sp1-deficient ES cells grow normally under standard conditions, the question arose whether Sp1 might be essential for the cell to cope with cellular stress situations like induced DNA damage. Previously, it has been found that Sp1 knock out ES cells are not hypersensitive to UV radiation when compared to wildtype cells (M. Marin, unpublished results). Here, we show that $Sp1^{-/-}$ ES cells are hypersensitive to ionising radiation. Interestingly, when $Sp3^{+/-}$ and $Sp3^{-/-}$ ES cells were subjected to the colony assays (not shown), $Sp3^{-/-}$ ES cells showed no increased sensitivity, whereas Sp3+/- ES cells showed an intermediate sensitivity. This has been seen for two independent heterozygous as well as two independent homozygous Sp3 knockout cell lines (see below).
Electroporation of human Sp1 cDNA in $Sp1^{-/-}$19 ES cells gave rise to several ES clones expressing the Sp1 transgene at different levels. The clone with the highest expression (about one half to one third of heterozygous Sp1 levels) has been analysed in colony assays in order to check its ability to rescue the ionizing radiation hypersensitive phenotype of its mother clone. However, no full rescue could be obtained. Rescue68 cells show only at low radiation doses a normal sensitivity, whereas at a dose of 8 Gy increased sensitivity is observed, which is probably due to the low expression level of the Sp1 transgene. However, the Sp1 expression level provided by the rescue construct, is obviously sufficient to allow these cells to contribute normally to murine development. Even normal appearing, chimeric males have been obtained, which were fertile, but did not give germ-line transmission. As one of these males was highly chimeric, this could mean, that the expression level of Sp1 in rescue68 cells is high enough to sustain their survival within a chimeric animal, but not high enough to allow these cells contribute to the germline. However, the number of two animals is too small to substantiate this suggestion.

14-3-3σ has been identified in DNA chip experiments comparing RNA from $Sp1^{+/-}$ and $Sp1^{-/-}$ ES cells as a gene that is dramatically down-regulated in $Sp1^{-/-}$ ES cells (see Chapter2 and this chapter). Indeed, Northern blot analysis confirmed reduced 14-3-3σ RNA levels in $Sp1^{-/-}$, and surprisingly also in $Sp3^{-/-}$ ES cells. Both cell lines show virtually no detectable 14-3-3σ protein level.
As the mouse sequence has become available, we could analyse the putative promoter region of the mouse 14-3-3σ gene (Ensembl Transcript ID ENSMUST 00000041945).

Surprisingly, within a fragment of 9 kb upstream of the mouse 14-3-3σ coding region no Sp1 binding site, neither a GC-box (GGGCGGG) nor a GT-box (GGTGTGGGG) could be found. However, in the human 14-3-3σ locus (Pubmed AF029081) a GT-box and an inversed GC-box (CCCGCCC) is found within 100 bp upstream of the transcription start-site. In the mouse locus, the sequence of the GT-box is changed to (GGTGTGGCG). Differences for p53 binding sites can be found as well. The human 14-3-3σ gene carries two p53 binding sites within 2 kb of its regulatory 5'-region (Hermeking et al., 1997). However, no p53 consensus sequence (RRRCWWGYYYRRRCWWGYYY; R = purine, Y = pyrimidine, W = A or T; (el-Deiry et al., 1992)) can be found within the 9 kb of upstream regulatory region of the mouse 14-3-3σ gene. So far, no Sp1/Sp3 influence on 14-3-3σ expression has been reported. The different availabilities of GC- and GT-boxes as well as p53 binding sites in mouse and human however raise the question if 14-3-3σ transcription is regulated in different ways in the two species. The lack of Sp1 binding sites in the mouse 14-3-3σ promotor region could mean, that unidentified Sp1/Sp3-dependent regulatory elements further upstream (enhancer elements) are incapacitated in $Sp1^{-/-}$ and $Sp3^{-/-}$ ES cells. Sp1 and Sp3 have often been shown to equally activate transcription of a gene. It might as well be possible that Sp1/Sp3 work indirectly on transcriptional and/or post-transcriptional control of 14-3-3σ. Possibly, in the Sp1 KO ES cells an as yet unidentified transcriptional regulator that activates 14-3-3σ transcription is down-regulated. Furthermore, one can also speculate that Sp1/Sp3 regulate a factor that is responsible for post-transcriptional regulation of 14-3-3σ RNA or protein. Recently, Efp has been identified in mice as a RING-finger dependent ubiquitin-ligase (E3) that targets 14-3-3σ for proteolysis (Urano et al., 2002). Influences of Sp1 on the post-transcriptional level of 14-3-3σ could help to explain the robust protein expression in rescue68 cells compared to the rather low RNA levels in these cells.

Forced expression of a 14-3-3σ transgene could not rescue the ionizing radiation hypersensitivity of two independent $Sp1^{-/-}$ ES cell lines. This might be easily explained if 14-3-3σ is not the (only) crucial factor that is responsible for the phenotype of $Sp1^{-/-}$ ES cells. The observation, that $Sp3^{-/-}$ ES cells have severely reduced 14-3-3σ levels but are not hypersensitive to irradiation could also be explained in this way. However, an explanation for the intermediate irradiation sensitivity of $Sp3^{+/-}$ cells is difficult. Highly speculative, due to double-edged properties of Sp3 as activator, but also as putative repressor of gene transcription (e.g. (Hagen et al., 1994)) a reduced expression of Sp3 protein in Sp3 heterozygous cells would not influence its activating properties. Rather it would disturb its repressive function in gene regulation so that repressive effects of Sp3 can be overruled by Sp1 activity. This could disturb the balance of the group of genes that regulates the cellular answer to DNA damage.

14-3-3σ has been described as a major regulator of the G2/M checkpoint that exerts its control by sequestration of the cdc2 (=cdk1) kinase in the cytoplasm after DNA damage, thereby preventing the onset of mitosis before DNA damage has been repaired (Chan et al., 1999; Hermeking et al., 1997). However, we were unable to see gross cell cycle abnormalities in $Sp1^{-/-}$ ES cells after irradiation, despite them having severely reduced 14-3-3σ levels. Western blot analysis of the cellular distribution of the cdc2 kinase did not reveal abnormal distribution of this protein. These differences might be explained in the use of different cell types or cells from different species. The above mentioned studies were carried out in human colorectal cancer cells, which are differentiated epithelial cells. 14-3-3σ has been the only p53 target gene detected in $Brca1^{-/-}$ ES cells by DNA chip analysis being down-regulated (Aprelikova et al., 2001). Indeed, $Brca1^{-/-}$ ES cells were shown to have increased numbers of cells in G2/M-phase after DNA-damage, implying a role for 14-3-3σ in G2/M arrest of ES cells as well. However, the p53 dependency of Brca1 overexpression on the induction of 14-3-3σ has only been shown with a human 14-3-3σ promoter construct in human cancer cells (Aprelikova et al., 2001). Taking into account that no p53 binding sites could be detected in the mouse 14-3-3σ promotor (see above), the question remains, how Brca1 co-activates the mouse 14-3-3σ in a p53 dependent manner.

Pluripotent embryonic stem cells have unusual cell cycle properties. For example, triple knock out ES cells of pRB and its relatives p107 and p130 that are known to contol the G1/S phase transition do not show altered growth characteristics (Dannenberg et al., 2000) It has also been shown that wildtype ES cells do not undergo p53-dependent cell cycle arrest after DNA damage, but undergo p53-independent apoptosis (Aladjem et al., 1998). Recently, 14-3-3σ has been connected to apoptosis (Samuel et al., 2001) as it has been shown to counteract premature apoptosis in human colorectal cancer cells after DNA damage through sequestration of the pro-apoptotic protein Bax in the cytoplasm. It might be of interest, if the down-regulation of 14-3-3σ in Sp1-deficient cells alters their apoptotic behaviour after DNA damage.

14-3-3σ has been a very interesting candidate gene to explain the observed ionizing radiation hypersensitivity of $Sp1^{-/-}$ ES cells. Indeed, we could show that 14-3-3σ is a target gene of Sp1 in ES cells, but at the moment we can neither support nor deny a crucial role for 14-3-3σ in the described radiation hypersensitive phenotype of $Sp1^{-/-}$ ES cells, the reason of which stays elusive.

Colony assay after irradiation

Sensitivity of ES cells to ionising radiation was determined by measuring their ability to form colonies after irradiation. Briefly, cells were trypsinized, counted and seeded in triplicate in various numbers on gelatinised 60mm dishes. After 12-24 hours the cells were irradiated with a ^{137}Cs source. Cells were grown for 7 days, fixed and stained, before colonies were counted.

Northern Blot analyses

10 µg of total RNA was separated on 1% agarose gel containing 0.02 M MOPS [3-(N-morpholino)propanesulfonic acid], 5 mM sodium acetate, 1mM EDTA and 2.2M formaldhyde and blotted on to nylone membranes and hybridised under standard conditions (Sambrook). The 14-3-3σ Northern probe was generated by PCR amplification from E14 cDNA using the sense primer (5'-GCCTTTCCCAAACCCTGAAT-3') and the antisense primer (5'-ACCTCCTGGATTATTCGGAT-3'), which lie within the 3'-untranslated region of the 14-3-3σ gene and yield a PCR product of 226 bp length.

Preparation of nuclear and cytoplasmic protein extracts

Extracts were prepared using a hypotonic lysis followed by a high salt extraction of nuclei according to (Andrews and Faller, 1991). ES cells were trypsinized and pelleted. Pellets were washed once with cold PBS. Cell pellets were resuspended in 400 µl cold buffer A (10mM HEPES-KOH pH7.9, 1.5mM $MgCl_2$, 10 mM KCl, 0.5mM dithiothreitol, 0.2mM PMSF). During the following incubation on ice for 10 minutes the cells swell and the cell membranes were fragmented by the following vortexing of the samples for 10 seconds. Samples were centrifuged for 1 minute at 4°C. The supernatants containing the cytoplasmic and membrane cell fractions were separated and stored at –80°C. The pellets were resuspended in 50-100 µl of cold buffer C (20mM HEPES-KOH pH7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM dithiothreitol, 0.2mM PMSF) and incubated on ice for 20 minutes (high salt extraction). Debris was removed by centrifugation of the samples for 2 minutes at 4°C. The supernatant fraction containing the nuclear proteins were stored at –80°C.

Protein concentrations of nuclear and cytoplasmic fractions were measured at 562 nm using the BCA protein assay reagent (Pierce).

Western analysis

For nuclear and cytoplasmic extracts 50µg of protein were separated on 10% SDS-polyacrylamide gels and blotted on PDVF membranes (Immobilon-P, Millipore) by

electroblotting. Blots were incubated in blocking solution (3% BSA, 0.05% Tween20 in PBS) for 1 h at room temperature. Incubation with the primary antibody (Sp1: gift from G. Suske, Marburg, TFIIH p62: gift from S. Winkler, Rotterdam, 14-3-3σ: Santa Cruz Biotechnology sc-7683, cdc2: Santa Cruz Biotechnology sc-54) was done for 1 h at room temperature at a 1:1000 dilution in fresh blocking solution. Blots were washed six times for 10 minutes in washing solution (PBS containing 0.05%Tween20). After subsequent incubation with an appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature, blots were washed six times for 10 minutes before bands were visualized using chemiluminescence.

Construction of cDNA vectors

Sp1 rescue vector

Human Sp1cDNA was isolated as SpeI-SmaI-fragment from pSp1-778c (gift from J.T. Kadonaga, San Diego) and cloned into the SmaI site of PGK-CAS (gift from J. Essers, Rotterdam) in order to express Sp1 cDNA under the control of the PGK promoter.

14-3-3σ rescue vector

14-3-3σ was amplified by PCR from mouse ES cell cDNA using the sense primer (5´-GGTATCGATAAGCTTGCTAGCATGGAGAGAGCCAGTCTGATC-3´) and the antisense primer (5´-ACCTCCTGGATTATTCGGAT-3´). The sense primer introduced a NheI site directly in front of the start codon, the antisense primer contains a BamHI site. The resulting PCR product of 1074 bp included a 306 bp fragment of the 3´-UTR of 14-3-3σ and was cloned into the vector pGEM-T (Promega). Several clones of transformed bacteria were sequenced. One containing the correct sequence was used to isolate the 1kb NheI-BamHI-fragment, which was cloned into the NheI-BamHI-digested pTZ19xSacI (gift from R. Rottier, Rotterdam), thereby introducing a HA-tag at the 5´-end of 14-3-3σ. HA-14-3-3σ was isolated as EcoRI-BamHI fragment and cloned into the SmaI site of pPGK-CAS in order to express 14-3-3σ cDNA under the control of the PGK promoter.

Cell culture and blastocysts injection

The linearized Sp1 rescue construct as well as the linearized 14-3-3σ rescue construct were used for co-electroporation of ES cells together with a linearized plasmid carrying the puromycin selectable marker (pHA263pur, gift from G. Weeda, Rotterdam). For the Sp1 rescue construct only Sp1-/-19 cells were electroporated, whereas for the 14-3-3σ rescue construct Sp1+/-20/8 as well as Sp1-/-19 and Sp1-/-20 were transfected.

ES cells clones were selected with puromycin (0.7mg/ml). For the Sp1 rescue the integration of the cDNA construct was confirmed by Southern blot analysis and its expression by western blotting (Sp1 antibody was a gift from G. Suske, Marburg)
For the 14-3-3σ rescue, construct expression was confirmed by western blotting using a primary HA-antibody (gift from D. Meijer, Rotterdam).
From obtained Sp1-/-19 ES cells that express the Sp1cDNA construct, rescue68 ES cells were injected into C57Bl/6 host blastocysts. Embryos were isolated at E9.5 and stained for lacZ expression.

X-gal staining of embryos

Embryos from blastocysts injection were isolated at E9.5, washed in PBS and fixed for 1 hour at room temperature in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA, 0.02% NP-40. After fixation embryos were washed in PBS containing 0.02% NP-40, before being stained overnight at room temperature in a solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 and 1mg/ml X-gal (Sigma). After staining embryos were washed in PBS and photographed.

Cell cycle analysis

One day before cells were exposed to irradiation, 3 x 10^6 cells were plated on a 10 cm dish per cell line and timepoint. The next day, cells were irradiated with a ^{137}Cs source at a dose of 8 Gy. At the times indicated, samples were washed twice with PBS before trypsin-treatment. In PBS resuspended cells were filtered through a 35µm cell strainer (Becton Dickinson). Again cells were collected by centrifugation before being resuspended in 0.4 ml PBS and fixed by addition of 4 ml ice-cold 70% ethanol. Until staining with propidium iodide (PI), samples were kept at 4°C. For PI staining, ethanol-suspended cells were centrifuged, the ethanol was decanted from the cell pellets, cell pellets were suspended in 5 ml PBS. After centrifugation and removal of the PBS, cells were suspended in 1 ml PI staining solution (0.1% (v/v) Triton X-100 in PBS, containing 0.2 mg/ml DNAse-free RNase A and 0.02 mg/ml PI) and incubated for thirty minutes at room temperature before analysis on the FACS cytometer.

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Chapter 4

A conditional knock out allele of the gene encoding transcription factor Sp1

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Abstract

The lethal phenotype of mice with conventional gene targeted alleles of the transcription factor Sp1 prevents the analysis of its in vivo functions beyond embryonic day 10. By use of the Cre-loxP system, two conditional knockout (cKO) alleles of Sp1 were generated carrying the selection cassette in two different transcriptional directions. When these mice were mated to mice carrying a ubiquitously expressed Cre transgene under the control of the chicken β-actin promoter, one of these alleles resulted in embryonic lethality at day 10. This allele did not show any phenotype in compound Sp1cKO/Cre mice when Cre was expressed specifically in Schwann cells of the nervous system and sertoli cells of the testis under the control of the Desert hedgehog (Dhh) promoter. However, Sp1cKO mice that express Cre specifically in erythrocytes under the control of the μ-LCR (locus control region) and promoter of the β-globin gene, die around embryonic day 12. Thus, lineage-specific Sp1 conditional knockout embryos reveal an important role for Sp1 in erythroid cells but not in Schwann cells or Sertoli cells. In addition, a mouse line ubiquitously expressing a fusion protein of Cre and the ligand binding domain (LBD) of the estrogen receptor was used to test the possibility of rendering Cre action dependent on the binding of the estrogen agonist tamoxifen. Both, tamoxifen application on murine embryonic fibroblasts derived from these mice and the prolonged application of tamoxifen directly to the mice resulted in very low recombination frequencies and therefore proved ineffective for the activation of Cre recombinase in the majority of cells within an organ.

The transcription factor Sp1 is the founding member of a large family of transcription factors characterized by their highly conserved DNA binding domain, consisting of three zinc fingers of the Cys_2-His_2-type (for review see (Bouwman and Philipsen, 2002; Philipsen and Suske, 1999)). Many ubiquitously, as well as tissue-specifically, expressed genes contain GC/GT-boxes, the DNA binding sites of this transcription factor family (Crossley et al., 1996; Hagen et al., 1992; Imataka et al., 1992; Kingsley and Winoto, 1992; Letovsky and Dynan, 1989) in their regulatory regions. Sp1 itself is ubiquitously expressed (Saffer et al., 1991) and has been implicated in the regulation of several hundred putative target genes.

Inactivation of the murine Sp1 gene results in embryonic lethality at around day 10 of gestation (E10) with severely retarded embryos showing a broad range of abnormalities (Marin et al., 1997). However, ES cells with both Sp1 alleles disrupted grow normally under standard cell culture conditions.

To circumvent the restrictions of the conventional knockout, which prevents analysis of later developmental stages, we generated a conditional knockout allele of Sp1 by use of the Cre-loxP system. This system takes advantage of the Cre recombinase from bacteriophage P1 which excises DNA sequences flanked by two of its 34bp short recognition sites, so-called loxP sites, when arranged in head-to-tail orientation (Sauer, 1998).

Two loxP sites were inserted into the Sp1 locus, flanking the last two exons that code for the zinc finger DNA binding domain and the C-terminal domain of Sp1. In addition, the sequences flanked by loxP comprise a neo-selection marker surrounded by intervening sequences of the β-globin gene. We generated two conditional knockout (cKO) alleles of Sp1, which differ in the orientation of the selection cassette. The cKO construct containing the selection cassette in the same transcriptional direction as the Sp1 gene appears to be a hypomorphic allele and resulted in embryonic lethality. The homozygous embryos express a reduced amount of Sp1 and show an interesting phenotype of head malformations, possibly due to transcriptional interference and/or premature poly-adenylation. The cKO construct with transcriptional direction opposite to that of the Sp1 gene resulted in viable and apparently normal mice when homozygous. These mice were used for crossings with ubiquitously, as well as tissue-specifically, Cre expressing mice in order to delete the Sp1 sequences between the two loxP-sites. The crossing of the Sp1cKO mouse line with the CreCAG mouse line, that expresses Cre under the ubiquitously active chicken β-actin promoter, gives rise to an embryonic lethal phenotype, similar to but distinct from the phenotype seen in the conventional knock out.

The use of the Dhh-Cre mouse line, that expresses Cre tissue-specifically in the Schwann cell in the nervous system and Sertoli cells in the testis, showed that Sp1 is not essential for

the development and function of these cell types. Crosses with the erythroid-specific PEVCre line, that expresses Cre under the control of the μ-LCR (locus control region) and promoter of the β-globin however, result in an embryonically lethal phenotype. Taken together, these Sp1 conditional knockout studies show that Sp1 plays an important role in the erythroid lineage during midgestation.

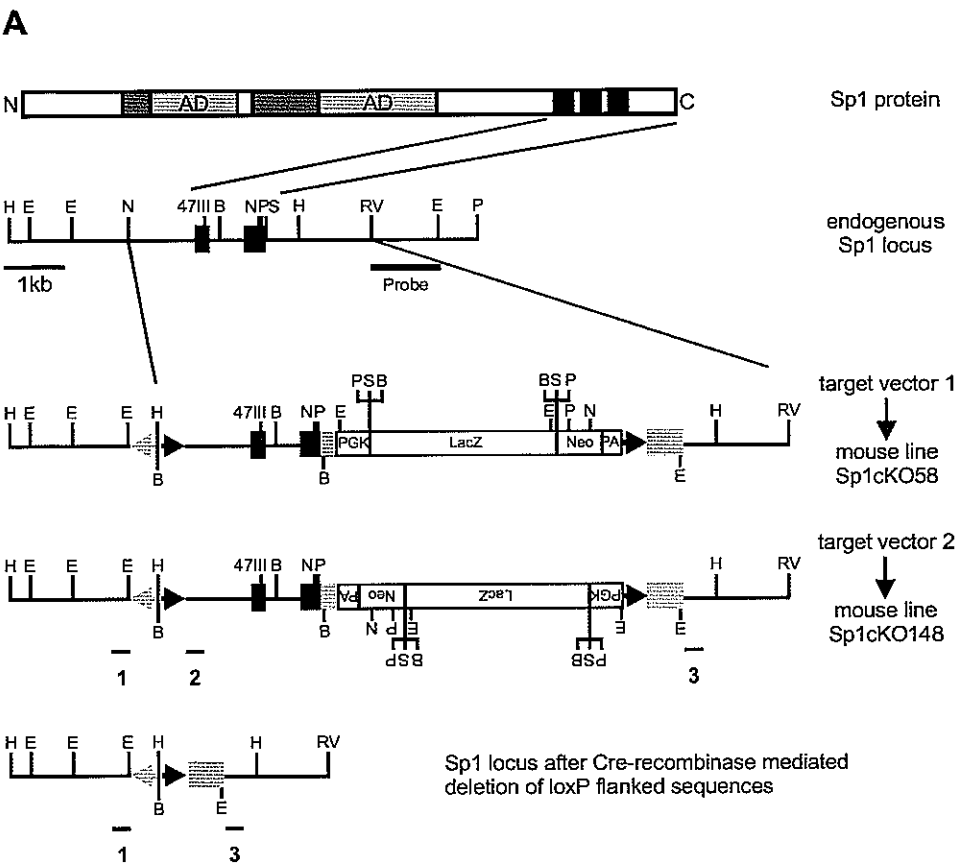
In order to gain control over the action of the Cre recombinase, we made use of mice that express the Cre recombinase fused to a mutated form of the estrogen ligand-binding domain. This fusion renders the action of Cre dependent on the binding of the artificial ligand 4-hydroxy-tamoxifen (4-OHT). However, mice expressing the Cre-LBD fusion protein under the control of the ubiquitously active cytomegalovirus (CMV) promoter show disappointingly low, almost undetectable frequencies of recombination, even at prolonged exposure to tamoxifen-containing drinking water. Also murine embryonic fibroblasts isolated from compound CMVCreTAM/Sp1cKO embryos and exposed to 4-OHT in cell culture only show extremely low recombination frequencies. Taken together, these observations question the use of tamoxifen-dependent activation of Cre in systems where Cre needs to be activated in a high percentage of the cells in an organ.

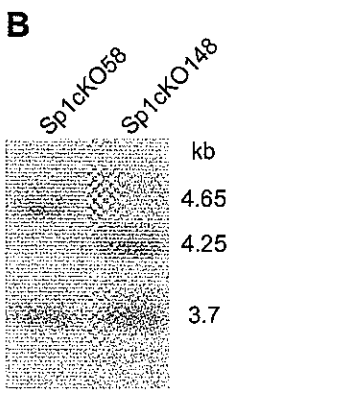
Results

Constructs and ES cells

The endogenous murine Sp1 locus was manipulated by introducing two loxP-sites flanking the two last exons of the Sp1 gene that code for the zinc fingers and the C-terminus of the protein. Upstream of the 5'-loxP site, an FRT site was introduced in order to have a possibility of introducing sequences such as additional exons. As a selection cassette, a lacZ-neo-fusion gene under the control of the PGK promoter was introduced downstream of the last exon. As the sequence of the 3'-untranslated region of Sp1 was unknown, the selection cassette was flanked by IVSII sequences (derived from intron 2 of the human β-globin gene) with the aim to create an artificial intron for the removal of the selection cassette from the Sp1 RNA by splicing. Two different targeting vectors were constructed containing the selection cassette in either direction, to minimize the risk of inadvertent transcriptional interference and/or aberrant poly-adenylation.

Cre-induced recombination of the two loxP-sites would result in the deletion of the intervening sequences, thereby leading to expression of a 65kDa truncated form of Sp1 that lacks the DNA binding domain and the C-terminal part of the protein. The deletion of these domains effectively results in an Sp1 *null* mutation (Marin et al., 1997).





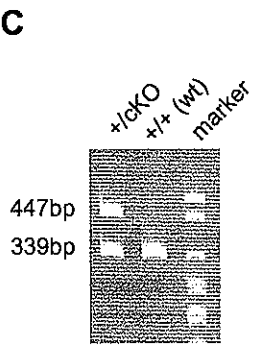


Figure 1: (A) Sp1 protein, endogenous locus, floxed locus and recombined locus after Cre action. All elements are drawn to scale with the exception of the loxP sites (black arrow), the FRT site (grey arrow), the intervening sequence of the β-globin gene (grey boxes) and the primers 1, 2, 3. Black boxes in the protein depict three zinc fingers, whereas in the genomic locus they depict the last two coding exons. Explanations: H, HindIII; E, EcoRI; B, BamHI; 47III, Eco47III, N, NcoI; P, PstI; RV, EcoRV; pA, polyadenylation signal; PGK, PGK promoter. (B) Southern blot of PstI digested genomic ES cell DNA using the probe shown in (A). (C) PCR analysis of genomic ES cell DNA using primer combination 1 and 2 depicted in (A).

Both constructs were linearized with NotI and electroporated into male murine E14 ES cells. After 10 days of selection in G418 containing medium, clones were picked and analysed for homologous recombination of the targeting construct into the endogenous locus by Southern blot and use of an external 3'-probe (Figure 1A).

Out of 145 picked clones for targeting construct 1, 15 clones showed correct recombination (targeting efficiency 10%) at the 3' end (Figure 1B), but only 2 of those also carried the 5'-loxP site (total targeting efficiency 1.4%). For the targeting vector 2, 211 clones were picked, 29 of them showed the expected band (targeting efficiency 14%) at the 3' end (Figure 1B). Of those 29 clones, 6 clones also contained the 5'-loxP site (total targeting efficiency 2.8%). The existence of the 5'-loxP site was tested by PCR (Figure 1C) using primer 1 and primer 2 depicted in Figure 1A.

Expression of Sp1 from the conditional knockout alleles

Total RNA was isolated from several different ES cell lines, and Northern blot analysis using a 400 bp probe spanning the zinc fingers and the C-terminal part of human Sp1 cDNA was performed in order to compare the expression levels of Sp1 (Figure 2A).

Endogenous Sp1 RNA was detected as an 8.2kb band. The Sp1 knockout ES cell line Sp1-/-20 expresses only the N-terminal part of Sp1, as the zinc fingers are deleted by this allele (Marin et al., 1997). Therefore the probe cannot detect this truncated Sp1 RNA.

As a control, RNA from the rescue68 cell line, a Sp1 knockout cell line expressing a transduced human Sp1 cDNA, shows a transcript with an estimated size of 2.9 kb.

In the conditional knockout cell lines Sp1cKO58 and Sp1cKO148, a new RNA band arises that can be explained by transcriptional read-through, yielding an RNA molecule with the size of 13.1 kb. This RNA species is hardly detectable in the Sp1cKO58 cell line, whereas it is readily visualized in the Sp1cKO148.

Expression of Sp1 protein by the conditional KO allele was further tested by electroporating a Sp1 knockout targeting vector carrying a hygromycine selection marker (Marin et al., 1997) into Sp1cKO148. Clones were picked and analysed for the genotype KO/cKO by Southern blot. Figure 2B shows a western blot probing for Sp1 protein in nuclear extracts of ES cell lines. The Sp1KO cell line shows the truncated Sp1 protein, missing the zinc fingers and the C-terminal domain. The full-length protein in the rescue68 ES cell line is hardly visible, whereas it is readily detectable in the Sp1cKO148/KO cell line. As this full-length protein is originating from the cKO allele it can be concluded that Sp1 is expressed approximately at normal levels from the cKO148 allele. The Sp1cKO148/KO cell line has also been subjected to a colony assay to analyse sensitivity to irradiation as described in chapter 3. Sensitivity to irradiation was comparable to Sp1 wildtype or Sp1 heterozygous ES cell lines (data not

shown), suggesting that the expression level of Sp1 from the cKO allele is sufficient to sustain its physiological functions.

Chimeras

ES cells containing the correctly floxed Sp1 locus were karyotyped and one clone for each targeting construct, Sp1cKO58 for targeting construct 1 and Sp1cKO148 for targeting construct 2 (Figure 1A), was injected into C57/bl6 blastocysts. Chimaeric male mice were used for further breeding and transmitted the floxed Sp1 allele (conditional knockout allele, cKO) to their offspring (F1 generation). F1 animals were intercrossed in order to obtain mice homozygous for the cKO alleles.

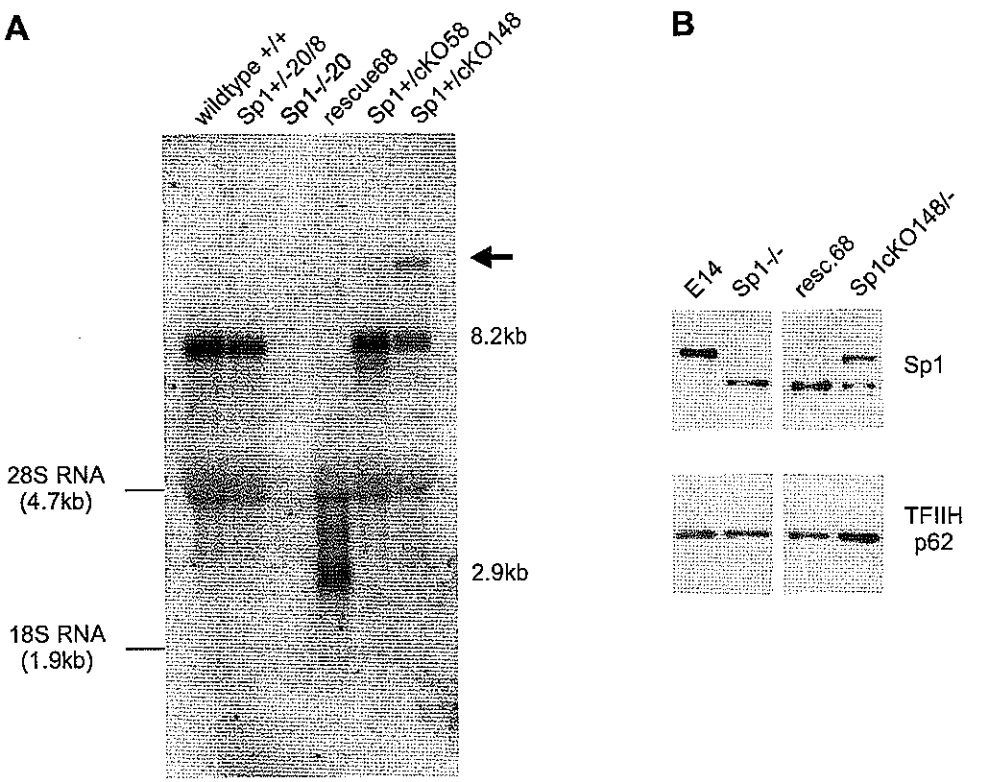


Figure 2: (A) Northern blot analysis of ES cell RNA with a 400bp BamH1/NcoI fragment from human Sp1cDNA as probe which contains the zinc finger and C-terminal domain of Sp1. Rescue68 is a Sp1KO ES cell clone transfected with human Sp1cDNA. The RNA species specific for the cKO clones is marked with an arrow. (B) Western blot analysis using a Sp1-specific antibody on nuclear ES cells extracts prepared 5 hours after irradiation of the cells with 5 Gy.

Sp1 conditional knock out mice

Homozygous mice carrying the selection cassette in opposite orientation to the Sp1 transcriptional direction (Sp1cKO148, see Figure 1A) were obtained, appeared normal and were fertile. They showed the expected Mendelian ratio (Table1).

genotype	number of mice	ratio	expected ratio
+/+	11	1 : 3.5	1 : 4
+/cKO	17	1 : 2.2	1 : 2
cKO/cKO	10	1 : 3.8	1 : 4

Table 1: Genotype distribution of pups born from heterozygous Sp1cKO148 crossings. A total number of 38 pups from 5 litters were used for genotyping.

However, for the Sp1cKO58 mouse line, carrying the selection cassette in direct orientation to the transcriptional direction of the Sp1 gene, no viable homozygous Sp1cKO mice were obtained from heterozygous crossings.

Genotyping of embryos during earlier developmental stages revealed, that homozygous cKO58/cKO58 embryos die around E12. These embryos show apparently normal development of different tissues and organs like heart, limbs, and somites, whereas severe deformations of the forebrain and face are observed. In the most extreme cases, all face and forehead structures are missing (Figure 3 C, left panel). More typically, these structures were severely reduced in size (Figure 3 C, right panel). Protein extracts from E10.5 Sp1cKO58 embryos were analysed for Sp1 protein levels (Figure 3A) and showed a reduced Sp1 expression level in cKO/cKO embryos compared to +/cKO embryos. We were interested to see whether a high expression level of Sp1 could be detected in head structures of embryos that could be correlated with the observed phenotype of the homozygous SpcKO58 embryos. For this purpose we made use of the conventional heterozygous Sp1 deficient mice.

Heterozygous Sp1KO embryos express β-galactosidase as a fusion with the N-terminal part of the Sp1 protein, thereby mirroring the expression of Sp1 (Marin et al., 1997). As can be seen in Figure 4, LacZ expression is readily detectable in the neuroepithelium of the head and the neural tube. Also, lacZ expression is shown in the neuroectoderm structures of the eye (Chapter 5, Figure 3). Taken together, these data suggest that the development of head and facial structures requires a minimal expression level of Sp1 that is not reached by the cKO58 allele.

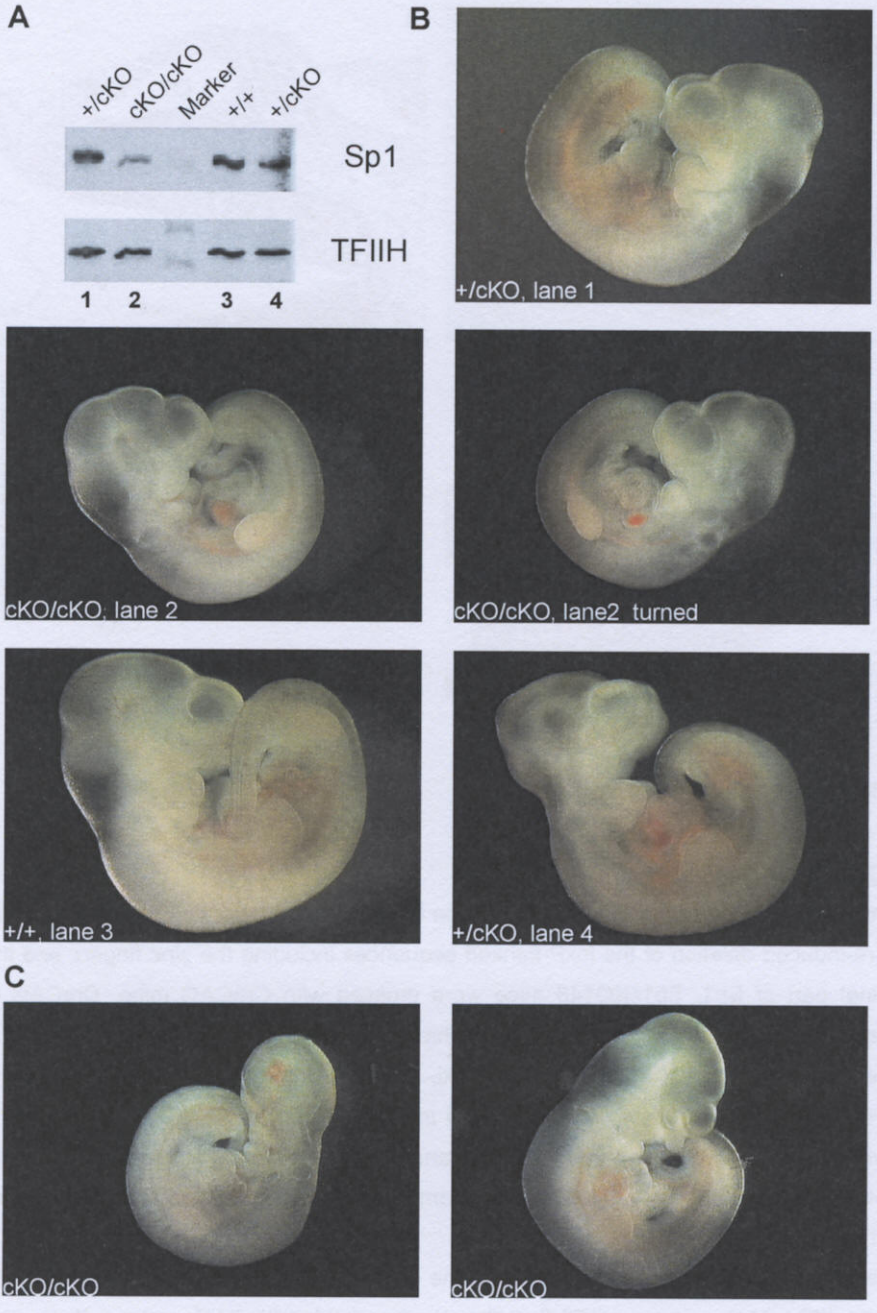


Figure 3: (A) Sp1 Western blot on nuclear extracts from Sp1cKO58 embryos on E10.5. The same blot was reprobed with TFIIH antibody as loading control. (B) Photos of the embryos used for western blot in (A). (C) Additional examples of Sp1cKO58 embryos of E10.5 that show severe forehead deformations.

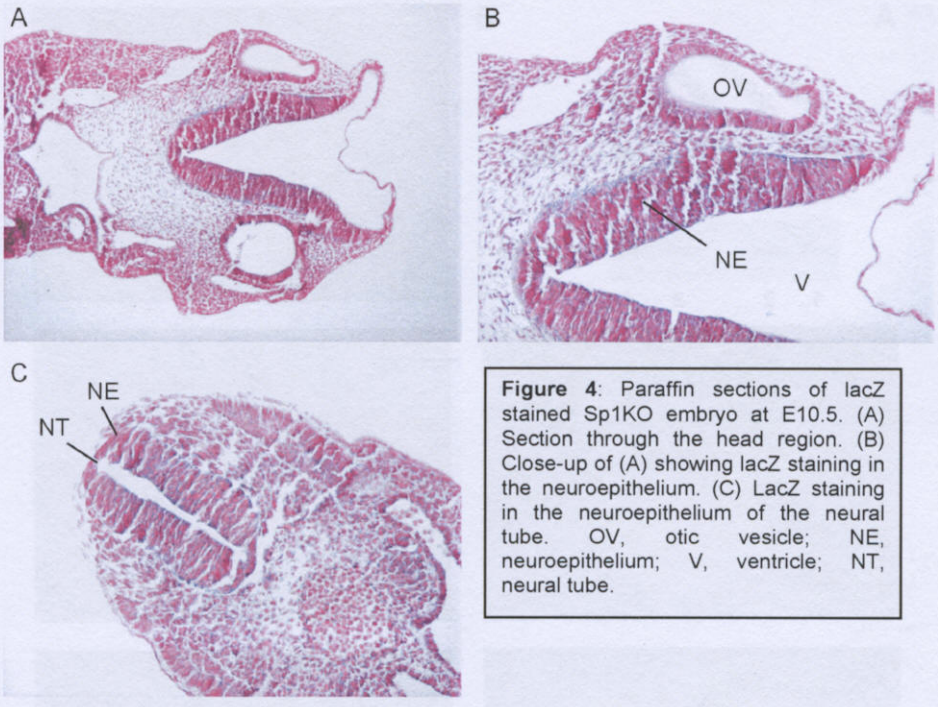


Figure 4: Paraffin sections of lacZ stained Sp1KO embryo at E10.5. (A) Section through the head region. (B) Close-up of (A) showing lacZ staining in the neuroepithelium. (C) LacZ staining in the neuroepithelium of the neural tube. OV, otic vesicle; NE, neuroepithelium; V, ventricle; NT, neural tube.

Cre induced knock outs of Sp1

CreCAG/Sp1cKO148 mice

In order to test, if the Sp1cKO148 allele can be transformed to a conventional knockout allele by Cre-induced deletion of the loxP-flanked sequences including the zinc fingers and the C-terminal part of Sp1, Sp1cKO148 mice were crossed with CreCAG mice. CreCAG mice express the Cre protein under the control of chicken β-actin sequences, which render the Cre expression ubiquitous (Sakai and Miyazaki, 1997). As embryos homozygous for the conventional knockout allele die around E10 *in utero* (Marin et al., 1997), compound mice carrying two cKO alleles and the CreCAG transgene are expected to die around the same developmental stage due to deletion of the same domains. The deleted cKO allele is further referred to as 'del' allele.

Indeed, no compound mice were born with the del/del//CreCAG genotype. Figure 5A shows a del/del//CreCAG embryo at E9.5. Although the del/del//CreCAG embryo died prior to dissection, it still is further developed than the KO/KO embryos at the same stage of development described in Marin et al (1997). Detection of the recombined del allele, the cKO and the wildtype allele was done by PCR using the three primers depicted in Figure 1A. Figure 5B shows an example of such a genotyping PCR from tail biopsies of live born

Sp1cKO148/CreCAG mice. The use of these primers can give rise to three different PCR products: a 447bp product for the cKO allele, a 339bp product for the wildtype allele and a 313bp product for the del allele. As the mother of this litter has a cKO/cKO genotype, all the offspring should show this allele, except if they are compound for the CreCAG transgene. Indeed, the mouse that showed no cKO PCR product (lane 4), also carried the Cre transgene. As lane 3 shows the genotype del/cKO of an alive-born and apparently normal mouse, this is a further indication that the expression level of the Sp1cKO148 allele is sufficient for normal murine development.

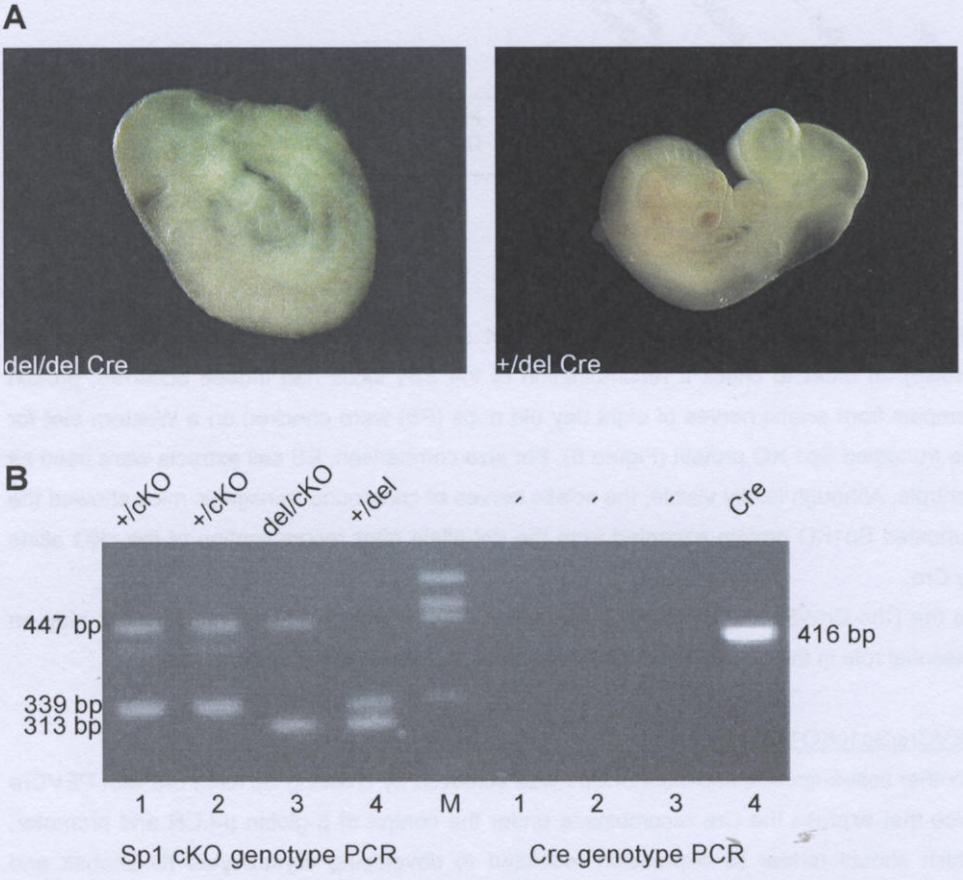


Figure 5: (A) CreCAG/Sp1cKO148 embryos at E 9.5. (B) Example of a genotyping PCR from tail biopsies of a litter of CreCAG/Sp1cKO148 mice. The genotypes of the parents were +/del//heterozygous CreCAG (father) and cKO/cKO (mother). All the offspring show the cKO band except for mouse 4. As this mouse expresses Cre recombinase, the Sp1cKO allele has been deleted.

Dhh-Cre/Sp1cKO148 mice

Dhh-Cre mice express the Cre recombinase under the control of the Desert hedgehog (Dhh) promoter and were a gift from Martine Jaegle and Dies Meijer (Erasmus MC Rotterdam).

Dhh is expressed in Schwann cells of the nervous system, vascular endothelium, endocardium and in Sertoli cells of the testis (Bitgood et al., 1996).

As we were interested in the outcome of tissue-specific deletions of Sp1, we crossed Dhh-Cre transgenic mice with Sp1cKO148. The resulting compound mice, carrying the Sp1cKO allele homozygously and the Dhh-Cre transgene, were born at the expected Mendelian ratio with no apparent phenotype. cKO/cKO male mice, carrying the Dhh-Cre transgene were fertile.

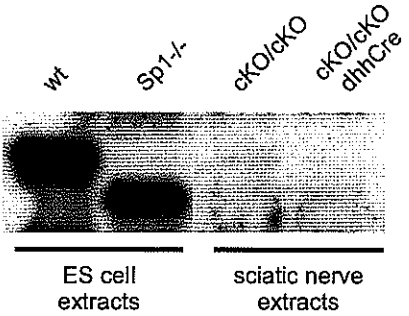


Figure 6: Sp1 western on protein extracts from Dhh-Cre/Sp1cKO148 pups at P8.

Myelination of isolated sciatic nerves did not show any obvious abnormalities (data not shown). In order to check if recombination of the Sp1 locus had indeed occurred, protein samples from sciatic nerves of eight day old pups (P8) were checked on a Western blot for the truncated Sp1 KO protein (Figure 6). For size comparison, ES cell extracts were used as controls. Although faintly visible, the sciatic nerves of compound transgenic mice showed the truncated Sp1KO protein expected from the del allele after recombination of the cKO allele by Cre.

As the Dhh-Cre//Sp1cKO/cKO mice are apparently normal, Sp1 does not seem to play an essential role in the development and function of Schwann cells and Sertoli cells.

PEVCre/Sp1cKO148 mice

Another tissue-specific knockout of Sp1 was achieved by crossing Sp1cKO148 with PEVCre mice that express the Cre recombinase under the control of β-globin μ-LCR and promoter, which should render its expression restricted to developing erythrocytes (D. Drabek and T. de Wit, unpublished results). We used PEVCre line C for our experiments.

No mice were born with the genotype PEVCre//Sp1cKO/cKO. Dissection of embryos revealed that compound embryos die around E12 of gestation. Genomic DNA of liver, as an erythropoietic organ and heads, as controls from embryos at E12.5 was prepared to check for the deletion fragment. In the liver DNA from compound transgenic embryos, the deletion fragment could easily be detected (Figure 7A, samples 5-8).

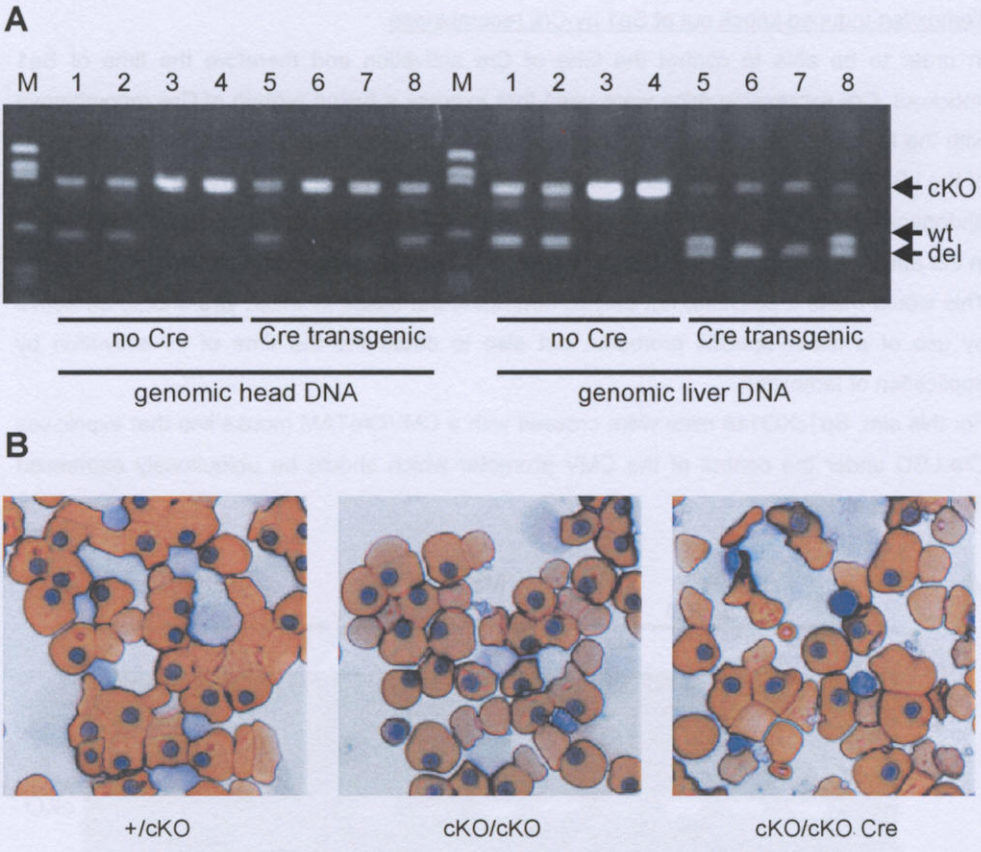


Figure 7: (A) PCR on genomic DNA from head or fetal liver of PEVCre/Sp1cKO148 embryos at E12.5 showing the Sp1 deletion fragment in the liver, whereas only trace amounts can be seen in the heads. (B) Cytospins of fetal blood.

Weakly visible deletion bands could also be detected in the genomic head DNA, suggesting that the expression of the PEVCre is slightly leaky or that the brain contains circulating blood cells. Fetal livers looked normal, although slightly smaller, and were red, suggesting ongoing erythropoiesis. However, the embryos were dying, looked pale with hardly any blood visible in the circulating system. Fetal blood was isolated and prepared as cytospin samples (Figure 7B) with subsequent staining. No obvious differences could be detected in samples from PEVCre//cKO/cKO (Figure 7B, right panel) compared to +/cKO (Figure 7B, left panel) samples. All samples show definitive fetal erythrocytes that have expelled their nucleus and contain similar amounts of hemoglobin as judged by their brown colour. This brown colour originates from O-dianisidine in the staining solution that is oxidised by hemoglobin, generating a brown chromophore (Liem et al., 1979).

Tamoxifen-induced knock out of Sp1 by Cre recombinase

In order to be able to control the time of Cre activation and therefore the time of Sp1 knockout, Cre expressing mice were used that express a fusion protein of Cre recombinase with the modified ligand binding domain (LBD) from the estrogen receptor. The modification of the LBD results in its high affinity binding to the estrogen agonist tamoxifen as opposed to endogenous estrogen as its natural ligand. The Cre-LBD fusion protein should stay inactive in the absence of ligand (tamoxifen) and should become activated in its presence.

This would make it possible, not only to determine the tissue in which Cre should be active by use of a tissue-specific promoter, but also to determine the time of its activation by application of tamoxifen.

For this aim, Sp1cKO148 mice were crossed with a CMVCreTAM mouse line that expresses Cre-LBD under the control of the CMV promoter which should be ubiquitously expressed (Brocard et al 1997).

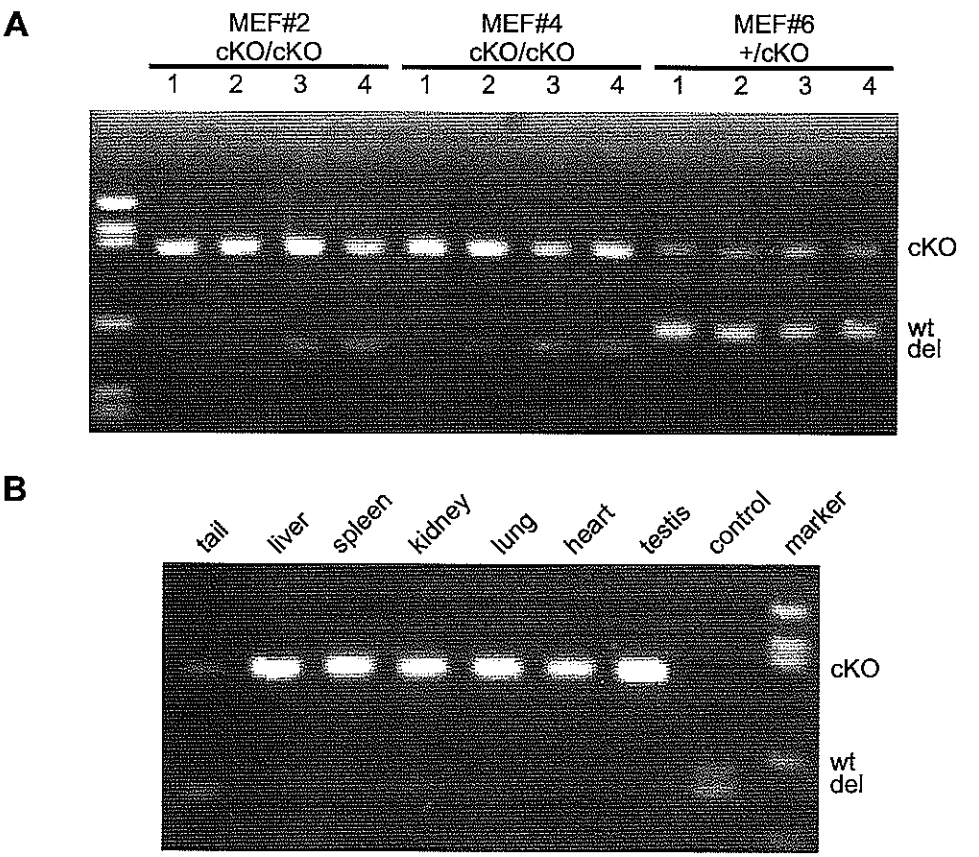


Figure 8: (A) PCR on genomic DNA from murine embryonic fibroblasts isolated from CMVCreTAM/Sp1cKO148 embryos at E13.5.All three MEF cell lines carry the CMVCreTAM transgene. Cells grown in 1, medium containing the solvent ethanol, 2, in 500nM 4-OHT for five days, 3, in 500nM 4-OHT for 2 passages, 4, in 500nM 4-OHT for 4 passages. (B) PCR on genomic DNA of isolated organs from a CMVCreTAM//Sp1cKO/cKO male mouse after 13 weeks of tamoxifen administration at a concentration of 0.1 mg/ml and 1% ethanol (v/v) in the drinking water. For comparison, the control shows the wildtype and the Sp1 deletion fragment of a CreCAG/Sp1cKO mouse.

Mice with the genotype CMVCreTAM//cKO/cKO were born, appeared phenotypically normal, and were fertile. From CMVCreTAM//+/cKO crossings embryos of E13.5 were used to isolate murine embryonic fibroblasts (MEFs). These were genotyped and grown either in the presence of 4-Hydroxytamoxifen (4-OHT) or the dissolvent ethanol only. After certain time points (see Figure 8A) genomic DNA was prepared and tested for recombination by PCR using the primers depicted in Figure 1A. Even in the presence of 500 nM 4-OHT during four passages, only a very faint Sp1 deletion fragment is detectable (Figure 8A), indicating a very inefficient activation of the Cre recombinase.

Drinking water containing tamoxifen at a final concentration of 0.1 mg/ml and 1% ethanol (v/v) was given to CMVCreTAM/Sp1cKO148 young adult mice. As the mice were still alive after 13 weeks of continuous tamoxifen administration, they were sacrificed, and genomic DNA was prepared from different organs.

	+/cKO/CMVCreTAM n=3	**cKO/cKO/CMVCreTAM n=3**
WBC ($x10^9$/l)	6.3 (±1.7)	6.5 (±0.9)
RBC ($x10^{12}$/l)	10.4 (±0.5)	10.7 (±0.2)
HGB (mmol/l)	9.7 (±0.1)	10.3 (±0.15)
HCT (l/l)	0.65 (±0.01)	0.69 (±0.01)
MCV (fl)	63 (±2.3)	65 (±0.6)
PLT ($x10^9$/l)	933 (±82)	932 (±33)

Table 2: Hematological values of CMVCreTAM/Sp1cKO148 mice after 13 weeks of tamoxifen administration in the drinking water. WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; PLT, platelets; l, litre; n, number of animals. Standard deviations are given in brackets.

PCR analysis of this genomic DNA revealed an almost complete failure of Cre activation as measured by Sp1cKO recombination. Figure 8B shows PCR products obtained from genomic DNA of a male CMVCreTAM//cKO/cKO mouse. Only the tail DNA showed the expected deletion fragment.

Blood of CMVCreTAM//cKO/cKO mice was obtained by orbital punctation and analysed. Hematological values were normal and no differences could be detected in CMVCreTAM//cKO/cKO mice compared to CMVCreTAM//+/cKO mice (Table 2).
Taken together, these results show a very inefficient activation of the Cre recombinase, far from the activation levels that were hoped for. This could be due to reduced recombinase activity and/or mosaic expression of the Cre-LBD fusion protein.

Discussion

In order to be able to investigate the function of the transcription factor Sp1 at developmental stages later than embryonic day 10, a conditional knockout of Sp1 was made by use of the Cre-lox system.
At the time of construction of the targeting vector, the genomic organisation of the Sp1 gene was largely unknown. Therefore the manipulation of the sequence directly behind the last known coding region carried the risk that it would disturb the presumable 3'-untranslated region. Indeed, the now available whole-genome sequence information of the mouse shows that the 3'-untranslated region behind the SmaI site used for insertion of the selection cassette is 1.3kb long. In order to decrease possible unfavourable influences, a 5'-splice donor and a 3'-splice acceptor surrounded the inserted selection cassette. This was done to create an artificial intron. The intervening sequence was therefore to be removed by splicing. However, as can be seen from Figure 2A an aberrant transcript (arrow) longer than the wildtype RNA of 8.2kb is seen in both of the conditional knockout alleles. Therefore, it is most likely that the assumed splicing does not take place efficiently. Higher expression of Sp1 from the cKO148 allele compared to the cKO58 allele must be due to the transcriptional direction of the selection marker, possibly through mechanisms involving transcriptional interference and/or aberrant poly-adenylation, which may result in destabilized mRNA.

Whereas the cKO148 allele provides sufficient Sp1 transcription to sustain mouse development, cKO58 is a hypomorphic allele. The interesting head/brain phenotype of cKO58/cKO58 embryos at E10 (Figure 3) and the embryonic lethality are likely due to subcritical levels of Sp1 expression from the cKO58 allele. Another indication that the Sp1 expression level may be specifically crucial for neuroectoderm development comes from the lacZ expression pattern of heterozygous Sp1KO embryos at E10.5 (Figure 4). LacZ expression in these mice mimics Sp1 expression (Marin et al., 1997). The neuroepithelium of the head, as well as the neural tube, show marked lacZ expression.

Interestingly, hypomorphic alleles of other brain-related transcription factors have been described to result in phenotypes resembling the head/brain phenotype described above. For example, sole expression of a hypomorphic Otx2 allele results in mutant embryos that do not develop forebrain structures like telencephalic vesicle and diencephalon as well as eyes, also showing failure in neural tube closure. At E18.5, the head is missing from the nose upward (Tian et al., 2002). Mice homozygous for the small eye allele, a hypomorphic allele of Pax6, show forebrain patterning effects (Stoykova et al., 1996).

In contrast to Sp1cKO58 mice, homozygous Sp1ckO148 mice develop to term, are apparently normal and fertile. Therefore these mice could be used for the mating with Cre expressing mice in order to test whether Sp1 is crucial for the development of certain tissues. The apparently normal development of compound Dhh-Cre//Sp1cKO/cKO mice suggests that Sp1 is not essential for the development of Schwann cells in the nervous system or for Sertoli cells in the testis.

However, erythropoietic depletion of functional Sp1 protein in Sp1cKO148 mice expressing Cre recombinase under the control of a β-globin μ-LCR and promoter, results in embryonic death around E12. At this time, the fetal liver is the main organ of erythropoiesis. Compound embryos show normal livers, although slightly smaller. Cytospins of fetal blood however display no gross abnormalities, as both immature erythrocytes that still contain their nucleus as well as enucleated fetal erythrocytes are found. Earlier studies on the expression of embryonic hemoglobin genes at E8.5 in embryos of the conventional Sp1 knockout (Marin et al., 1997) revealed lower, yet still significant levels of expression compared to wildtype embryos. The PEVCre//Sp1cKO/cKO embryos will be of interest to study the influence of Sp1 on erythropoiesis and the expression of fetal hemoglobin genes.

Our attempt to activate Cre protein by administration of tamoxifen was unsuccessful. However, several reports have been published that show successful temporal activity control of the Cre-LBD fusion protein in cells as well as in mice (Feil et al., 1997; Imai et al., 2001a; Imai et al., 2001b; Li et al., 2000).

The Sp1 deletion fragment in the culture of murine embryonic fibroblasts was readily detectable by PCR after addition of 4-OHT. However, the efficiency was very low and even prolonged culture in the presence of 4-OHT of up to four passages could not increase the efficiency. A possible reason might be that the expression level of the Cre-LBD protein could be too low for efficient excision. However, at the moment we cannot exclude the possibility that Sp1 expression is vital for survival of MEFs and that cells which successfully recombine the Sp1 locus undergo apoptosis and therefore depleted from the culture. However, if this were the case, massive cell death and prolonged doubling times would have been observed.

The only tissue that showed some Sp1 recombination after administration of tamoxifen to mice was the tail. A highly reduced level of recombination was found in the lung. An earlier study using CMVCreTAM mice reported the highest recombination efficiencies in the tail (Brocard et al., 1997). However, other tissues also showed recombination. The Cre activation efficiencies achieved with our system were by far too low to be useful. Similar problems have been encountered with a transgenic mouse expressing the Cre-LBD fusion protein under the control of the β-globin μ-LCR and promoter (Lindeboom, 2002). It might be necessary to choose other activation mechanisms for Cre as have been recently established for example by expression of Cre under the control of a tetracycline-inducible promoter (St-Onge et al., 1996).

Besides the restraints of the tamoxifen-inducible Cre, the conditional Sp1 knockout allele described above can be used for further studies of tissue-specific functions of Sp1.

Materials and Methods

Construction of the targeting vectors

Genomic Sp1 DNA fragments originated from a 129 mouse cosmid genomic DNA library (Marin et al., 1997).

The 5'-part of the constructs was prepared by removal of the 5´-SalI-HindIII-fragment from the plasmid BS-C5 (-0.7 kb BamHI) and the insertion of the FRT-loxP sequence into the NcoI-site. The Eco47III-SpeI fragment thereof was replaced by the Eco47III-SpeI-fragment of BS-C5-C6 containing the rest of the Sp1 exons depicted in Figure 1. The resulting plasmid BS-C5-C6 (-1.7kb SalI-HindIII)-FRT-loxP was used for the generation of both targeting vectors.

For targeting vector1, a loxP site was inserted in the NotI site of the plasmid pPGKßgeobpA (Friedrich and Soriano, 1991) downstream of the polyA signal, whereas for the targeting vector 2 it was inserted in the opposite direction in the HindIII site in front of the PGK promoter. Fragments containing the loxP and the PGKßgeobpA-sequence were isolated and inserted into the SmaI site of pBS-ßIVS88 in order to surround these fragments with splice donor- and splice acceptor-sites from the ß-globin-gene. DNA fragments containing the 3´-loxP and the PGKßgeobpA surrounded by the ßIVS-sequences were inserted into the SmaI site of BS-C5-C6 (-1.7kb SalI-HindIII)-FRT-loxP directly behind the last Sp1 exon.

Transfection and analysis of ES cells

E14 ES cells were electroporated with 15 μg of NotI-linearized targeting vector1 or targeting vector2. Clones were selected with G418 (200 μg/ml). Homologous recombination was

analysed by Southern blotting of PstI-restricted genomic DNA using the external 1.1kb EcoRV-EcoRI probe flanking the right homologous arm (Figure 1). An internal lacZ probe was used to detect random integrations in the genome. The integration of the 5'-loxP was detected by PCR (see genotyping of mice).

Generation of chimeric and Sp1-conditional knock out mice

Two Sp1cKO targeted ES clones, carrying the PGK-lacZneo-cassette in two different orientations, were injected into C57Bl/6 host blastocysts. Chimeric males were mated to C57Bl/6 females and germline transmission was obtained. The F1 offspring were interbred to expand the stocks and to obtain mice carrying the conditional allele homozygously.

Northern blot analysis

Total RNA was prepared from ES cell cultures with TRI REAGENT (Sigma) according to the manufacturer's instructions and subjected to denaturing gel electrophoresis and Northern blotting following standard procedures. A 400bp BamH1/NcoI fragment isolated from human Sp1 cDNA (gift from J. Kadonaga, University of California, San Diego) was used as probe. This fragment spans the coding sequence of the zinc finger and C-terminal domain of Sp1 that is deleted in Sp1KO cells.

Western blot analysis of ES cells

Nuclear extracts were prepared from ES cells 5 hours after irradiation with 4 Gy using a hypotonic lysis followed by a high salt extraction according to (Andrews and Faller, 1991). Protein concentrations of extracts were measured at 562 nm using the BCA protein assay reagent (Pierce). For the further procedure see 'western blot analysis of sciatic nerves'. The TFIIH p62 antibody was a gift from S. Winkler, Rotterdam.

Histological analysis

Sp1+/- embryos (Marin et al., 1997) were collected at the appropriate stages, washed in PBS and fixed for 1 hour at room temperature in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA, 0.02% NP-40. After fixation embryos were washed in PBS containing 0.02% NP-40, before being stained overnight at room temperature in a solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 and 1mg/ml bluogal (Sigma). After staining embryos were washed in PBS and kept in 4% formaline saline at 4°C overnight for post-fixation, washed with PBS and processed to paraffin. 7-µm paraffin-sections were counterstained with Nuclear Fast Red and mounted with Entellan (Merck).

Genotyping of embryos and mice

Genomic DNA was prepared from heads (embryos) or tail biopsies (mice) and analysed by PCR.

Detection of Sp1 alleles: wt-cKO-del

The sense primer 1 (5´-GAGGCCTTGTTGCAAAAGTAAG-3´) and the antisense primer 2 (5´-ACACCACCAGATTCAAAGACTCT-3´) flank the FRT-loxP-sequence inserted into the 5´-NcoI-site of the locus. PCR was done for 30 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min). PCR product sizes were 339 bp for the wildtype allele and 447 bp for the cKO allele.

For the detection of the deletion fragment after Cre action the above mentioned primers were used in combination with an additional antisense primer 3, lying directly downstream of the SmaI site used to generate the targeting vectors. Whereas primer combination 1 and 2 detects the wildtype and cKO alleles mentioned above, primer combination 1 and 3 detects a deletion (del) fragment of 313 bp. PCR conditions were the same as described above.

Detection of Cre transgenic embryos or mice

For detection of Cre transgenic mice sense primer (5´-ACCCTGTTACGTATAGCCGA-3´) and antisense primer (5´-CTCCGGTATTGAAACTCCAG-3´) were used to generate a PCR product with 416 bp. PCR was done for 30 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min).

Tamoxifen administration in mice

Stock solution of tamoxifen citrate salt (Sigma) was prepared in 100% ethanol at a concentration of 10 mg/ml. This stock solution was used to prepare drinking water for mice with a final tamoxifen concentration of 0.1 mg/ml. The water was refreshed weekly.

Isolation of murine embryonic fibroblasts

E13.5 embryos from timed pregnancies of CMVCreTAM/Sp1cKO148 mice were isolated. Head, limbs and inner organs were removed. Remaining embryo pieces were washed in PBS, minced and cultured in MEF medium (DMEM medium: F10 medium (both Gibco BRL) 1:1, supplemented with 10% fetal calf serum, penicilline-streptomycine and non-essential amino acids (Gibco-BRL). Tamoxifen was administered after the cells had gone through crisis.

Tamoxifen administration in cell culture

For cell culture 4-hydroxy-tamoxifen (4OH-T) was dissolved in ethanol. Cells were incubated at a final concentration of 500 nM or in the presence of the solvent ethanol alone.

Hematological analysis

Blood samples from mice were collected by orbital bleeding and analysed on a F800 microcell counter.

Cytospins

Cells from isolated embryonic blood were prepared on slides by cyto-centrifugation at 500 rpm for 5 minutes. Slides were air-dried and subsequently stained with 1% O-dianisidine (Sigma) in methanol and a stain similar to Giemsa (Diff. Quick Red and Blue, Dade Diagnostika) (Beug et al., 1982).

Western blot analysis of sciatic nerves

Sciatic nerves were isolated and homogenized in 1x Sample buffer (0.125 M Tris-HCL, pH6.8, 20% glycerol, 4% 2-mercaptoethanol, 4% SDS, 0.005% bromephenolblue) by sonication on ice. Samples were boiled for 5 minutes prior to loading on a 10% SDS-polyacrylamide gel and subsequent blotting on PDVF membranes (Immobilon-P, Millipore). Blots were incubated in blocking solution (3% BSA, 0.05% Tween20 in PBS) for 1 h at room temperature. Incubation with the Sp1 primary antibody (gift from G. Suske, Marburg) was done for 1 h at room temperature at a 1:1000 dilution in fresh blocking solution. Blots were washed six times for 10 minutes in washing solution (PBS containing 0.05%Tween20). After subsequent incubation with anti-rabbit peroxidase-conjugated secondary antibody (DAKO, P0448) for 1 h at room temperature, blots were washed six times for 10 minutes before bands were visualized using chemiluminescence.

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Chapter 5

Sp1 heterozygosity in mice increases the incidence of ocular abnormalities in the C57Bl/6 background

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Abstract

Correct eye development depends on timely and spatially-correct expression levels of transcription factors and inductive signals. The mouse has been an important model to study the genetic basis of eye defects. Interestingly, a small percentage of C57Bl/6 inbred laboratory mice shows congenital ocular defects like microphthalmia, anophthalmia and cataracts. Here we report a dramatic increase of the frequency of such eye defects in C57Bl/6 mice lacking a full dose of the transcription factor Sp1. This is the first phenotype detected so far in the Sp1 heterozygous mice. Further results demonstrate that lacZ marker expression in ocular tissues of such Sp1 heterozygous mutant embryos indeed supports the idea that Sp1 is important for the development of the eye.

Introduction

During vertebrate ocular development, tissues from different embryonic origins interact in a timely and spatially-coordinated fashion to give rise to the different components of the adult eye (Graw, 1996). This process depends on the correct expression pattern of transcription factors and on a series of inductive signals between the interacting tissues. Any defect in this complex program of induction and differentiation of cells can result in congenital sight-threatening anomalies.

The prenatal development of the ocular system in the mouse becomes first evident at around embryonic day 8 (E8), when the optic placodes are formed as flattened areas of the neuroectoderm on either side of the presumptive forebrain region. The optic placodes enlarge and approach the overlying surface ectoderm as optic vesicles. The resulting close contact between these two tissues is required to induce the surface ectoderm at around E9.5 to thicken and become the lens placode, while the outer surface of the optic vesicle invaginates to form the optic cup. The inner layer of the optic cup will develop into the neuroretina while the outer layer will form the retinal pigmented epithelium. The developing eye stays connected with the brain via the optic stalk. Axons from the ganglion cells of the neural retina will grow to, and down, the degenerating optic stalk and form the optic nerve, connecting the eye with the visual centres of the brain. The lens vesicle is completely closed by E11 and separates from the surface ectoderm. The still close contact to the lens vesicle induces the overlying surface ectoderm to differentiate into the multi-layered cornea. The lens epithelial cells at the anterior side of the lens vesicle maintain their mitotic activity, whereas the posterior cells differentiate to lens fiber cells producing crystallins.

It is known that the genetic make-up of a mouse strain can influence the severity of a phenotype induced by artificial genetic modifications like the gene targeted deletion of a gene (Montagutelli, 2000).

Inbred C57Bl mice are known to develop spontaneous congenital eye abnormalities at low frequencies (Cook and Sulik, 1986; Kalter, 1968; Pierro and Spiggle, 1967; Pierro and Spiggle, 1969). Described abnormalities are microphthalmia and anophthalmia with a reported incidence varying between 4.4% (Chase, 1942) and 10% (Kalter, 1968). Corneal and/or lenticular opacities have also been described (Koch and Gowen, 1939) with a quite variable incidence. One or both eyes can be affected and a combination of anomalies, like corneal opacities in microphthalmic eyes can be found. The tendency of these inbred mouse strains to develop ocular abnormalities is thought not to be due to a strain-specific mutation but rather a propensity to manifest mutations induced by environmental influences (Smith et al., 1994).

Studies using chimeric mice showed that the ocular anomalies found in C57Bl/6 mice are based on retarded lens development (LoCascio et al., 1987), as C57Bl/6 cells showed a highly reduced contribution to the lens in comparison to other ocular tissues.

It was shown that lens development is retarded in all C57Bl/6 mice. The majority of adult mice are free from any apparent abnormalities, probably due to the ability of the embryos to compensate for environmental influences or not sufficiently severe defects (Robinson et al., 1993).

The eukaryotic transcription factor Sp1 contains three zinc finger motifs from the Cys_2-His_2-type and has been implicated in the regulation of many genes. It is ubiquitously expressed but its expression levels change during development and vary in different cell types, suggesting an important regulatory role of varying Sp1 expression (Saffer et al., 1991). Early work indicated that Sp1 binding sites in different genes show a broad range of binding affinities (Kadonaga et al., 1986). Differences in Sp1 levels are therefore likely to influence target gene transcription. Transcription of target genes with weak Sp1 binding sites will depend on higher Sp1 levels for robust transcription than those target genes with high affinity Sp1 binding sites. Sp1-deficient mouse embryos die around E10 of gestation, are severely retarded and show a broad range of abnormalities (Marin et al., 1997). Heterozygous animals were initially smaller than their wild-type littermates, but were otherwise found normal.

Here we report the increased incidence of ocular anomalies in Sp1 heterozygous knockout mice when crossed into the C57Bl/6 background.

Ocular abnormalities in Sp1+/- mice

During backcross of the heterozygous Sp1 knockout mice onto the C57Bl/6 background, a significant increase in the occurrence of eye abnormalities like microphthalmos and opacities was found in the heterozygous mutant mice compared to their wildtype littermates.

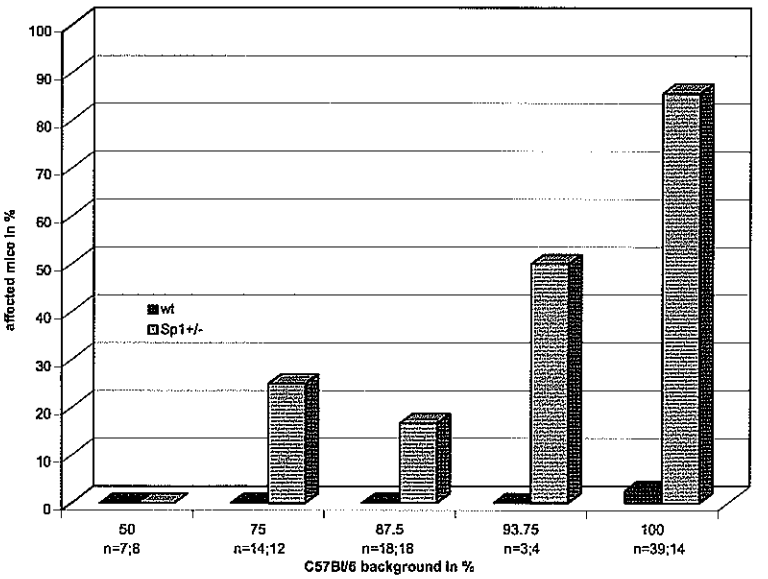

Figure 1: Increase of ocular anomalies in heterozygous Sp1 deficient mice with increasing proportion of C57Bl/6 background. Percentages of affected wildtype (wt) mice are shown as black boxes, percentages of affected Sp1 heterozygous animals are shown as grey boxes. n gives the number of animals that were checked for wildtype;Sp1 heterozygous genotype.

After more than 10 backcrosses heterozygous Sp1 deficient mice showed an 86% incidence eye abnormalities, whereas only 3% of their wildtype littermates were affected (Figure 1). Heterozygous mice from two independent Sp1 deficient lines behaved similarly (data not shown). Notwithstanding the general lower body weight found in all $Sp1^{+/-}$ mice, this is the only and the first specific phenotype detected in the heterozygous Sp1 knockout mice thus far. The occurrence of this phenotype is strictly dependent on the background, as a backcross to the (C57Bl/10 x CBA) mouse strain resulted in a total loss of the genotype-phenotype correlation detected in the C57Bl/6 background. With repeated cross to C57Bl/6 background, eye abnormalities reappeared again with increasing incidence, whereas no increase could be detected in the wildtype littermates.

At times, affected Sp1 heterozygous eyes showed a combination of opacity and reduced eye size. From a total number of 28 eyes from 14 Sp1 heterozygous mice of the C57Bl/6 background (see Figure 1), 13 eyes were normal, while 7 showed opacities. Four anophthalmic eyes were found (absence of an eye with only closed eyelids). Two eyes were very small (microphthalmos) as compared to the average eye size, and two eyes showed a combination of small size and opacity.

Histological examination of adult $Sp1^{+/-}$ eyes in C57Bl/6 background

In order to analyse the histological background of the opacities, an adult eye from a $Sp1^{+/-}$ mouse in the C57Bl/6 background was dissected. Figure 2 shows a persistent lens stalk as result of an incomplete detachment of the lens vesicle from the surface ectoderm. This leads to a persistence of the lens-cornea connection. Cataract formation can result from other abnormalities like alterations of lens tissue that change the light refraction index. However, we find that the cataracts are closely associated with the persistent lens stalks. As microphthalmia accounts for a high percentage of abnormalities and the incidence of opacities does not increase with age (not shown), a developmental defect like a persisting lens stalk is more likely to be the cause of the observed eye phenotype than a degenerative lesion of lens tissue.

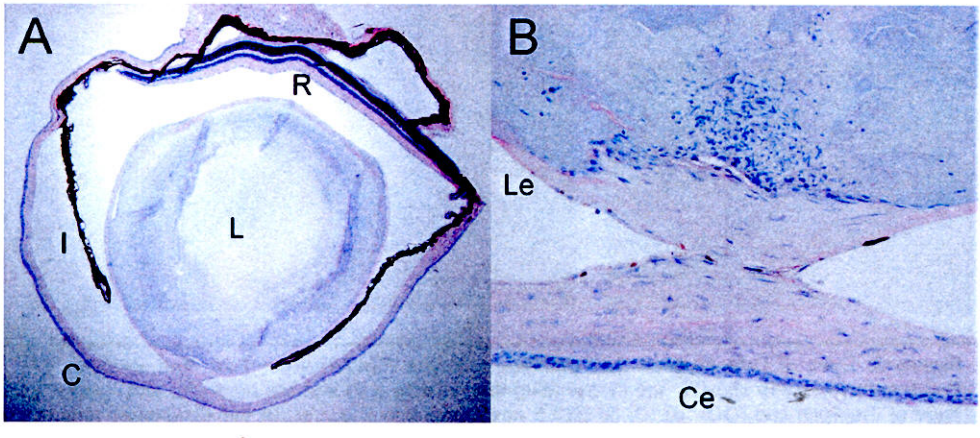

Figure 2: Eye of a C57Bl/6 adult heterozygous Sp1 deficient mouse showing a persistent lens stalk (hematoxylin/eosin stained). (A) Whole eye showing normal anatomy except for the lens-corneal bridge. (B) Close-up of lens-corneal bridge. L, lens; C, cornea; I, iris; R, retina; Le; lens epithelium; Ce, corneal epithelium.

Sp1 expression detected as lacZ expression in embryonic $Sp1^{+/-}$ eyes

As heterozygous Sp1 knockout mice express lacZ under the control of Sp1 regulatory elements (Marin et al., 1997), lacZ expression could be used to monitor Sp1 expression in

ocular tissues of heterozygous Sp1 deficient embryos. In addition to its ubiquitous expression, a surprisingly strong lacZ expression was detected in several ocular tissues (Figure 3) as well as in the developing head neuroectoderm, neural tube (see Chapter 4) and olfactory epithelium (not shown).

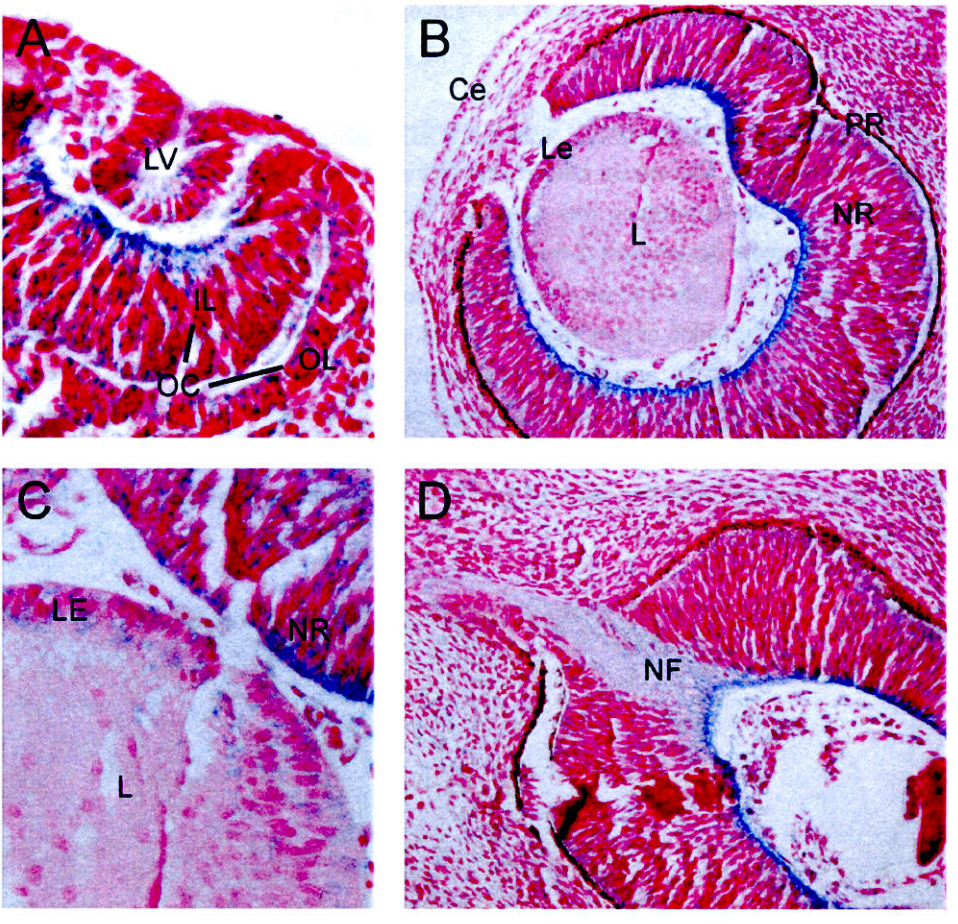

Figure 3: Eyes of heterozygous Sp1 deficient embryos (LacZ/Nuclear Fast Red staining). (A) E10.5 embryonic eye showing the invaginating lens vesicle (LV) and the inner (IL) and outer (OL) layer of the optic cup (OC). (B, C, D) E13.5 embryonic eye showing (B) the developing lens (L), lens epithelium (LE) and the two layers neural retina (NR) and pigmented retina (PR) of the developing retina. (C) A close-up shows the faint lacZ staining of cells in the lens epithelium (LE). (D) LacZ stained nerve fibres (NF) from ganglion cells of the neural retina within the optic stalk (the future optic nerve).

High levels of Sp1 in developing neural tissue have already been reported (Saffer et al., 1991). At E10.5 the lens vesicle, as well as both layers of the optic cup, show lacZ expression. The highest levels of expression are detected at the side of the developing neural retina that faces the invaginating lens vesicle (Figure 3A). It should be noted that the surface ectoderm shows higher expression in the area of the invaginating lens vesicle than in

the areas surrounding the vesicle. At E13.5, the highest expression levels still remain at the anterior border of the neural retina, whereas the inner region of the retina shows an even but lower expression (Figure 3B). An even lacZ expression pattern can also be seen in the lens epithelium, whereas expression is not detectable in the surface ectoderm that will differentiate into the corneal epithelium and in the future corneal stroma (Figure 3C). The axons of the ganglion cells of the retina that migrate to the optic stalk to form the optic nerve show especially profound lacZ expression (Figure 3B and 3D). Taken together, the incidence of eye defects in Sp1 deficient C57Bl/6 mice along with the Sp1 expression pattern suggest that Sp1 plays a role in normal eye development and maintenance.

Discussion

Here we report the first specific abnormality of heterozygous Sp1 deficient mice. In Sp1 deficient mice the penetrance of the macroscopically detectable eye opacities is strongly dependent on the C57Bl/6 contribution to the genome.

It is generally known that C57Bl/6 inbred mice are prone to congenital ocular defects like microphthalmia, anophthalmia and cataracts (Robinson et al., 1993). As a result of retarded lens development affecting all C57Bl/6 embryos, 5-15% of adult mice show these ocular anomalies. In our mouse colony we found 3% of wildtype animals that showed the described abnormalities, whereas 86% of their heterozygous Sp1 deficient littermates were affected (Figure 1). Within the C57Bl/6 background this enormous difference should be solely due to an Sp1 dosage effect, indicative of haploinsufficiency. In heterozygous Sp1 deficient ES cells a decreased level of Sp1 protein compared to wildtype ES cells has been detected in a gel retardation experiment (Marin et al., 1997).

We suggests that the phenotypic differences observed in $Sp1^{+/-}$ mice in different background strains are probably the effect of so-called modifier genes.

An increasing number of reports show that the genetic background of an inbred strain can modulate the phenotype of an introduced single gene mutation. This effect is attributable to the either direct or indirect cooperation between the causative gene and the modifier genes of the used inbred strain (Montagutelli, 2000; Nadeau, 2001). In our case, the Sp1 gene dosage may be important for the correct expression levels of one or more of these modifier genes in C57Bl/6 mice that influence eye development. Alternatively, Sp1 itself could be one of these modifier genes. As all C57Bl/6 mice are prone to eye anomalies at the developmental stage of lens invagination, this would mean that a reduced Sp1 level further deteriorates an already fragile balance of factors in this strain that influence ocular development at that stage. This then could result in the enormously increased incidence of

eye anomalies detected in adult $Sp1^{+/-}$ mice compared to their wildtype littermates. While the incidence increases dramatically in $Sp1^{+/-}$ mice, the range of anomalies we see in affected $Sp1^{+/-}$ mice is the same as was reported for affected wildtype C57Bl/6. We also see microphthalmic eyes, sometimes in combination with eye opacities as well as anophthalmia. Histological analysis of adult eyes showed that persistent lens stalks are the likely reason for the macroscopically observed opacities (Figure 2). These have also been described to contribute to ocular defects in C57Bl/6 mice (Cook and Sulik, 1986).

Several transcription factors are known to be involved in different stages of eye development (for reviews see (Ashery-Padan and Gruss, 2001; Graw, 1996). These include Otx2 (Zygar et al., 1998), Pax6 (Hill et al., 1991), Sox1/2/3 (Kamachi et al., 1998), Six3 (Loosli et al., 1999; Oliver et al., 1996; Oliver et al., 1995) and FoxE3 (Blixt et al., 2000).

It is tempting to speculate that the expression levels of one or several of these transcription factors are reduced in mice of the C57Bl/6 strain, leading to the described eye phenotypes. Furthermore, Sp1 could be directly or indirectly involved in the regulation of these transcription factors. This could explain the haploinsufficiency of Sp1 in C57Bl/6, as decreased Sp1 levels might further decrease the expression level of these transcription factors.

Pax6, for example, shows a similar expression pattern to Sp1 in some of the tissues that seem to be especially susceptible to reduced Sp1 expression levels. Pax6 is expressed in the developing brain, the developing eye, the neural tube and the olfactory epithelium (Walther and Gruss, 1991). As we have shown here ocular expression of lacZ in $Sp1^{+/-}$ mice (see Figure 3, for other expression sites see also Chapter 4, Figure 4), it would be interesting to perform studies in which the overlap of Sp1 and Pax6 expression in the eye is examined. Pax6 is known to play essential roles in the normal development of several organs including pancreas, central nervous system and the eye (Callaerts et al., 1997). Eye development shows a striking sensitivity to Pax6 gene dosage, as increased or decreased levels of Pax6 expression both lead to abnormal eye phenotypes. Overexpression in mice leads to severe abnormalities in the lens, ciliary body, retina, iris and cornea (Schedl et al., 1996). Reduction of Pax6 levels by heterozygous null mutations results in a number of anterior segment abnormalities in human eyes. Abnormalities like aniridia (iris hypoplasia), cataract, Peter's anomaly (persistent connection between lens and cornea), corneal opacities, adhesions between iris and lens and/or cornea and vascularization of the peripheral cornea have been described (Churchill and Booth, 1996; Hamburg, 1967; Hanson et al., 1994; Mirzayans et al., 1995; Ton et al., 1991). The corresponding anomaly in mouse and rat, known as the Small eye (Sey) mutation serves as a model of the human syndromes (Hill et al., 1991). It has been shown that lens placode formation is delayed in Sey heterozygous embryos leading to a severe reduction in the number of lens cells during early development (van Raamsdonk and

Tilghman, 2000). By generation of chimeric embryos, cell autonomous deficiencies of $Pax6^{+/-}$ cells in the lenses of chimeras could be detected. Heterozygous cells showed a significant underrepresentation in the lens placode at E10.5, with hardly any heterozygous cells detectable at E16.5. In contrast, $Pax6^{+/-}$ cells contributed normally to all other ocular tissues (Collinson et al., 2001). $Pax6^{-/-}$ cells are excluded from the surface ectoderm of chimeric embryos (Collinson et al., 2000). Persistent lens stalks were shown to be the result of autonomous deficiency in the lens, as chimeras did not show any lens-cornea bridge after E12.5, even when the corneal epithelium had a predominantly mutant composition. Tissue recombination experiments in rats using optic vesicles and surface ectoderm from $Pax6^{-/-}$ and wildtype embryos demonstrate that Pax6 has a cell autonomous role in the surface ectoderm but is not essential for the inductive capacity of the optic vesicle (Fujiwara et al., 1994). This is further supported by the finding, that tissue-specific depletion of Pax6 in the surface ectoderm impairs the formation of the lens placode (Ashery-Padan et al., 2000).
Despite its expression in different eye tissues including ciliary body, iris, cornea and retina, the lens is the only tissue that shows an overt sensitivity to Pax6 heterozygosity. Nevertheless, other eye tissues are also dependent on Pax6 expression. Further eye development in homozygous Sey mice ceases once the optic vesicle contacts the surface ectoderm, with no subsequent differentiation of ocular tissues. These mice also lack nasal cavities (Grindley et al., 1995; Hogan et al., 1986), exhibit brain abnormalities (Grindley et al., 1997; Schmahl et al., 1993; Stoykova et al., 1996) and die soon after birth.

The striking similarity between the C57Bl/6 background dependent $Sp1^{+/-}$ eye phenotype and the heterozygous Sey mouse phenotype, gives rise to the highly speculative hypothesis that Sp1 could be involved in the *in vivo* regulation of Pax6 expression. First, one could envisage, that C57Bl/6 in general have a slight reduction of Pax6 expression due to the action of modifier genes inherent to this mouse strain. This reduction could result in protein levels between the heterozygous and the wildtype Pax6 level. Given the fact that the eye and especially the lens are sensitive to Pax6 levels, this could result in an overall retarded eye development in C57Bl/6 mice due to impaired lens formation. In line with this argument are mosaic analyses of chimeric animals that showed, comparable to the fate of $Pax6^{+/-}$ cells, a selective exclusion of C57Bl/6 cells from the lens (Robinson et al., 1993). Despite the disadvantage of C57Bl/6 cells, most adult C57Bl/6 mice show near normal lens size and no gross eye abnormalities. However, dramatic changes are observed in mice with a reduced Sp1 level. In this population of C57Bl/6 mice the incidence of eye abnormalities reaches almost 90%. Assuming that Sp1 plays a role in the expression of modifier genes in C57Bl/6 mice that influence eye development, a reduction of Sp1 levels could interfere with the appropriate expression level of these genes and thereby, directly or indirectly, regulate Pax6

levels. Second, Sp1 could be directly involved in the regulation of Pax6 expression. Recently, it has been shown that the human Pax6 gene contains an enhancer element in exon 1 that can be activated by Sp1 (Zheng et al., 2001). Furthermore, the two Pax6 promoters P0 and P1 both contain Sp1 binding sites (Kammandel et al., 1999; Xu et al., 1999).

As Sp1 is a ubiquitously expressed transcription factor, its restricted expression in the neuroectoderm and its derivatives, like the developing retina and the nasal pit (not shown) of developing embryos, was surprising. Interestingly, a hypomorphic conditional knockout allele of Sp1 shows severe head malformations (see chapter 4). Here, we report that mice heterozygous for Sp1 show a higher incidence of ocular abnormalities in the C57Bl/6 background. It is reasonable to speculate that the observed phenotypes are correlated with the expression pattern of Sp1 during these developmental stages and in these tissues. As the heterozygous Sp1 mice still express relatively high levels of Sp1, the differences in transcriptional activation of target genes between wildtype mice and heterozygous mice are probably difficult to determine. The homozygous hypomorphic conditional knockout embryos express lower Sp1 levels and develop further than the homozygous Sp1 knockout embryos. However, they show a more drastic phenotype than Sp1 heterozygous mice and therefore might be of use for a target gene analysis at developmental points when Sp1 levels are obviously critical.

Materials and Methods

Scoring of eye abnormalities

Eyes were examined with a slit lamp or by normal examination as this turned out to give the same results.

Histological analysis of embryonic eyes

$Sp1^{+/-}$ embryos were collected at the appropriate stages, washed in PBS and fixed for 1 hour at room temperature in PBS containing 1% formaldehyde, 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA, 0.02% NP-40. After fixation embryos were washed in PBS containing 0.02% NP-40, before being stained overnight at room temperature in a solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 and 1mg/ml bluogal (Sigma). After staining embryos were washed in PBS and kept in 4% formaline saline at 4°C overnight for post-fixation, washed with PBS and processed to paraffin. 7-μm paraffin-sections were counterstained with Nuclear Fast Red and mounted with Entellan (Merck).

Histological analysis of adult eyes

Adult eyes were dissected and fixed overnight at 4°C in PBS containing 4% paraformaldehyde. Subsequently, samples were dehydrated via an alcohol series and embedded in paraffin. 4 µm microtome sections were mounted on slides and stained with hematoxylin and eosin according to standard procedures.

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Chapter 6

Concluding remarks and future outlook

Samenvatting

De cel is de kleinste eenheid die uit eigen vermogen levensvatbaar is. Deze eigenschap is gecodeerd in het genetisch materiaal van de cel, het DNA. Sommige van de informatie die is opgeslagen in het DNA (genen) wordt via een proces dat transcriptie genoemd wordt overgeschreven in een groep van relatief kortlevende moleculen, de messenger RNA's (mRNA's). De mRNA's zelf worden gebruikt om de uitvoerende moleculen van de cel, de eiwitten, te produceren. Eiwitten hebben veelvoudige functies in de cel. Het zijn bijvoorbeeld bouwstenen voor het skelet van de cel, of sensoren om de voedingsstatus van de cel te bepalen. Eiwitten kunnen ook reacties van de cel op een zich veranderende omgeving uitvoeren. Het geheel van eiwitten in een cel bepaalt de identiteit van een cel. Hersencellen hebben bijvoorbeeld een andere eiwitsamenstelling dan levercellen. Transcriptiefactoren zijn een groep van eiwitten die de productie van andere eiwitten regelen op het niveau van de eerste stap in de eiwitproductie, de transcriptie van het stuk DNA dat voor het eiwit codeert. Dit wordt bereikt door het herkennen en binden aan korte specifieke sequenties op het DNA die te vinden zijn in de regulerende gebieden van genen. Transcriptiefactoren interacteren met het RNA-polymerase, een eiwitcomplex dat de mRNA moleculen genereert, en regelen daarmee de hoeveelheid mRNA moleculen die voor een specifieke eiwit geproduceerd worden.

Dit proefschrift

Hoofdstuk 1 geeft een meer gedetailleerde overzicht van de hierboven genoemde processen en beschrijft de transcriptiefactor Sp1, het onderwerp van dit proefschrift, en aan Sp1 verwante eiwitten.

Sp1 (specificity protein 1) was een van de eerste geïdentificeerde zoogdier transcriptiefactoren. Sp1 is het eerste lid van een familie van transcriptiefactoren die verenigd zijn door het karakteristieke onderdeel waarmee ze aan DNA binden: drie zinc vingers.

Familieleden binden aan specifieke DNA sequenties, zogenoemde GC- of GT boxen, waarmee de belangrijkste basenparen in deze DNA sequenties aangegeven worden. Bindingsplaatsen voor de Sp1 familie van transcriptiefactoren bevinden zich in de regulerende gebieden van talrijke genen.

Omdat Sp1 in allen cellen van het lichaam aanwezig is, was het van belang om te zien of cellen zonder functioneel Sp1 eiwit levensvatbaar zijn. Daarom werden in ons laboratorium in embryonale stamcellen (ES cellen) van de muis DNA sequenties die voor Sp1 codeeren uitgeschakeld met behulp van een zogenoemde knockout benadering. Onverwacht zijn deze cellen in staat om zich verder te vermenigvuldigen. Wel sterven muizen die geen functioneel

Sp1 was among the first identified and cloned mammalian transcription factors (Dynan and Tjian, 1983; Kadonaga et al., 1987). Since then, an avalanche of data about biochemical and *in vivo* functions of Sp1 and other members of the Sp/XKLF family has been produced (Chapter 1).

In vivo functions of Sp1

Studies on Sp1 expression have shown that it is a ubiquitously expressed protein, although expression levels vary between different cell types and with differentiation status of cells (Saffer et al., 1991). Consistent with this broad expression spectrum, gene disruption of Sp1 results in embryonic lethality in mice around mid-gestation (Marin et al., 1997). Sp1-deficient embryos show a broad range of abnormalities. At this time of development organogenesis has started, as several developing organ systems can be identified, e.g. eye vesicles, heart, otic vesicles, somites and blood. Although, the phenotype of Sp1-deficient embryos is rather heterogeneous, none of these embryos survives after E10. Surprisingly, Sp1-deficiency is compatible with cellular survival as Sp1 null ES cells show normal proliferation characteristics under standard cell culture conditions ((Marin et al., 1997), this thesis). However, they are unable to contribute to normal murine development beyond E10, when injected into wildtype host blastocysts. As even support of surrounding wildtype cells *in vivo* does not result in the survival of Sp1-deficient cells, this inability to contribute to embryonic development after mid-gestation must originate from a cell-autonomous defect (Marin et al., 1997).

The embryonic lethality of Sp1-deficient mice precludes an analysis of the role of Sp1 in different organs and tissues at later ontogenic times. This problem can be circumvented by the generation of conditional knockout mice through the use of the Cre/lox- (Sauer, 1998; Sauer and Henderson, 1988) or Flp/FRT- (Vooijs et al., 1998) recombination systems. Tissue-restricted expression of the bacteriophage Cre- or the yeast FLP-recombinase results in tissue-restricted ablation of gene sequences that are flanked by the corresponding recognition sites loxP or FRT. These systems make it possible to study the function of widely expressed genes like Sp1 in distinct organs. We have applied the Cre/lox system to generate conditional knockout alleles of the Sp1 gene (this thesis).

In developing such a Cre/lox system to examine organ/tissue/lineage specific functions of Sp1, the choice of which organ/tissue/lineage to target resembles a game of chance. It is complicated by 1) the ubiquitous expression of Sp1 and 2) the possibility of functional redundancy between Sp1 and its ubiquitously expressed family member Sp3 (Hagen et al., 1992). In the studies presented in this thesis we limited our targeting to cells expressing Cre recombinase under the control of regulatory sequences from Desert hedgehog (DhhCre with

expression in endothelium, Sertoli and Schwann cells) and β-globin (PEVCre with expression in developing erythrocytes).

In generating the Sp1 conditional knockout alleles, we used a direct and reverse orientation of the selectable lacZ marker gene within the Sp1 locus. Whereas the reverse orientated allele allowed survival of homozygous conditional knockout mice (Sp1cKO148), the direct orientated allele led to an embryonically lethal phenotype of homozygous mice (Sp1cKO58) (Chapter 4).

Homozygous Sp1cKO58 die around E12. At E10.5 most embryos show severe forehead malformations, with sometime whole omission of forehead structures. By analysis of lacZ expression in Sp1 heterozygous embryos of the conventional knockout, high lacZ expression could be detected in neuroectodermal structures like the neuroepithelium of the head and the neural tube. As homozygous Sp1cKO58 embryos show reduced Sp1 protein levels, the Sp1 levels expressed from the cKO allele are probably too low to enable proper head formation. Another neuroectodermal structure, that shows high lacZ expression in Sp1 heterozygous knockout embryos, is the neuroretina of the developing eye (Chapter 5). As Sp1cKO58 show a phenotype that is probably correlated with the low Sp1 expression from the modified allele, it is conceivable that a gene expression analysis using DNA chips comparing homozygous Sp1cKO58 and wildtype littermates could reveal Sp1 target genes with disturbed expression in homozygous Sp1cKO58 embryos.

Previously, neural tissue of E8.5 and E12.5 embryos has been reported to express high levels of Sp1 (Saffer et al., 1991). Hence, it is of interest to specifically delete Sp1 in neuroectodermal structures by use of neuroectodermal specific Cre expression in the mice carrying the Sp1cKO148 allele.

Furthermore, in Drosophila, the Sp-related factor buttonhead is required for the development of the antennal, intercalary and mandibular segments of the head (Wimmer et al., 1993) and also in the formation of the peripheral nervous system (Wimmer et al., 1996). Expression of human Sp1 under the control of the buttonhead promoter partially rescued the buttonhead phenotype by support of the development of the mandibular segment in the head of buttonhead mutant embryos (Wimmer et al., 1993). Buttonhead has been shown to interact with the same TAFs (TBP-associated factors) as Sp1 (Schock et al., 1999). Hence, it is also of interest to examine whether buttonhead expression in homozygous Sp1cKO58 could ameliorate the head malformations in these embryos.

Two Cre-induced tissue-specific deletions of Sp1 using Sp1cKO148 mice were carried out (Chapter 4). Crossing the Sp1cKO148 mice with a transgenic Desert hedgehog (dhh) Cre mouse line (see above) resulted in no detectable phenotype. Compound mice were viable and fertile. This finding does not exclude that Sp1 plays a role in gene expression within the endothelial, Sertoli or Schwann cell types. However, it is possible, that it does not have vital

functions there. Another possibility would be, that Sp3 is able to take over Sp1 functions in these cell types. Sp3 has been shown to have very similar DNA-binding specificities (Hagen et al., 1992), to be able to activate many genes with a similar capacity than Sp1 (e.g. (Cogan et al., 2002; Galvagni et al., 2001)) and is ubiquitously expressed.

In contrast, compound mice of Sp1cKO148 and a PEVCre-transgene, which is expressed in developing erythrocytes, died *in utero* around E12. It could be shown that inactivation of Sp1 by deletion of its zinc finger domains efficiently takes place in the fetal liver, the main erythropoietic organ at that developmental stage. E8.5 embryos of the conventional Sp1 knockout express reduced, but still significant levels of the embryonic α-globin gene zeta and the embryonic β-globin genes βH1 and εy (Marin et al., 1997). Sp1 might play a role in regulation of the switching process of the globin gene expression from the embryonic-type genes to the adult-type genes (α1 and α2, βmajor and βminor) which takes place at this developmental stage. The knockout of EKLF, an erythroid-specific member of the Sp/XKLF family, results in defective erythropoiesis in the fetal liver (Nuez et al., 1995; Perkins et al., 1995) leading to severe aenemia due to a failure of adult β-globin gene activation. Many erythroid-specific genes contain binding sites for EKLF/Sp1 in their regulatory region (e.g. (Magness et al., 2000)) and both factors have been shown to physically interact with the zinc finger region of the erythropoietic transcription factor GATA-1 (Merika and Orkin, 1995). It could be possible that EKLF and Sp1 have overlapping, but also unique functions in the activation of erythroid-specific genes.

Rescue experiments were done with the aim to restore the cell-autonomous defect of Sp1-deficient cells in their ability to contribute to chimeric embryos. Transgenic Sp1 expression in originally Sp1-deficient ES cells (Chapter 3) resulted in highly chimeric embryos as detected by lacZ expression. Two adult chimeric males were obtained from these blastocysts injections. Whereas both were fertile, they did not give germ-line transmission. However, the number of animals studied was too small to confirm that the Sp1 expression level from the transgene is too low to sustain spermatocyte development. In the future, it might be interesting to specifically delete Sp1 in developing sperm cells (Ando et al., 2000; Vidal et al., 1998). The transcription of many spermatocyte specific genes depends on Sp1 binding sites (e.g. (Wilkerson et al., 2002; Zhang et al., 1999)) and increased Sp1 levels have been described in developing spermatids (Saffer et al., 1991).

Cellular functions of Sp1

As mentioned above, Sp1-deficient ES cells show normal proliferation under standard cell culture conditions (Marin et al., 1997). However, we showed that Sp1-deficient ES cells are hypersensitive to ionising radiation (this thesis). Sp1 expression from a Sp1 transgene only yielded about one half to one third of Sp1 levels in heterozygous deficient ES cells. Hence, it

appears that this expression level is not high enough to rescue the observed radiation hypersensitivity. For this reason it might be interesting to re-establish Sp1 expression through a knock-in approach, which should result in expression levels that are high enough to do so.

14-3-3σ, a gene that has been identified as marker protein for epithelial cells (Leffers et al., 1993; Prasad et al., 1992) has been shown to be down-regulated in Sp1-deficient ES cells (this thesis). 14-3-3σ overexpression has been described to induce G2 cell cycle arrest in human colorectal cancer cells (Hermeking et al., 1997), whereas in the presence of DNA damage 14-3-3σ deletion in these cells resulted in the loss of protection of damaged cells from entry into mitosis (Chan et al., 1999). Therefore, it was of interest to examine whether 14-3-3σ down-regulation in Sp1-deficient ES cells is involved in their hypersensitivity towards ionising radiation. This hypothesis is based on the observation that DNA damage in cells without 14-3-3σ results in mitotic entry prior to DNA damage repair. However, no gross differences in the cell cycle of Sp1-deficient and -proficient ES cells after irradiation were detected (this thesis). In addition, forced expression of 14-3-3σ in Sp1-deficient ES cells could not rescue their hypersensitivity towards ionising radiation. Recently, 14-3-3σ has also been implicated in the prevention of apoptosis after DNA damage (Samuel et al., 2001). Although again, these experiments were carried out in human colorectal cancer cells, it would be worthwhile to analyse whether Sp1-deficient ES cells undergo enhanced apoptosis following DNA damage.

Moreover, basically nothing is known about the function of 14-3-3σ in development. Therefore, it would be interesting to inject the 14-3-3σ-overexpressing $Sp1^{-/-}$ ES cells into recipient blastocysts to examine to what extent they can contribute to tissues of chimeric embryos. In this way, a potential role of 14-3-3σ as an important downstream effector of Sp1 activity during embryogenesis would be revealed. In addition, the 14-3-3σ-overexpressing Sp1 heterozygous ES cells could be used, in order to see whether 14-3-3σ overexpression itself is compatible with murine development.

As of now, the reason for the hypersensitivity to ionising radiation of Sp1-deficient ES cells is unknown. However, it is most likely due to diminished expression of genes involved in DNA repair. This might include genes encoding sensors of DNA damage, regulators of the DNA damage response and/or factors directly involved in DNA repair. As many of these genes are only up-regulated upon DNA damage, a microarray analysis could be performed by using RNA from irradiated wildtype- and Sp1-deficient ES cells in order to study whether Sp1 is involved in the induction of such genes after DNA damage.

Taken together, these studies on Sp1 show that Sp1 expression levels are critical for cells to enable them to react on the challenging insult of DNA damage and for proper development of

specific organs during embryogenesis. The definition of 'sufficient' Sp1 expression depends on the system that is studied.

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Summary

The cell is the smallest unit that can sustain its survival by its own. This ability of the cell is encoded by its genetic material, the DNA. Some of the information 'written down' in the DNA (genes) is transformed via a process called transcription into transitory molecules, the messenger RNAs (mRNA). The mRNA itself serves as template to produce the executive molecules of the cells, the proteins. Proteins can serve distinct functions in the cell. For example they build the cytoskeleton of the cell, sense the nutrition status of the cell and execute programs to enable the cell to react on changing conditions of its environment. The protein composition of a cell determines its identity. Brain cells have a different protein composition than liver cells. Transcription factors are a class of proteins that regulate the production of other proteins by regulating the first step in their formation, the transcription of the piece of DNA that encodes for the specific protein into the mRNA molecule. They do so by recognition of and binding to short specific sequences in the regulatory regions of genes. They interact with the RNA polymerase, the protein complex that actually produces the mRNA molecules, in order to regulate the amount of mRNA molecules produced.

Work described in this thesis

Chapter 1 gives an overview over the above mentioned processes in more detail and describes the protein of interest of this thesis, Sp1, and its relatives.

Sp1 (specificity protein 1) has been among the first identified mammalian transcription factors. It is the founding member of a family of transcription factors that are characterized by the module that enables them to bind to their specific DNA recognition site: three zinc fingers. Members of this transcription factor family bind to specific DNA sequences, GC- or GT-boxes, named after the predominant base pairs within this recognition site. Binding sites for this transcription factor family have been found in the regulatory region of numerous genes.

As Sp1 can be found in virtually all cells of the mammalian body, it has been of interest to determine if cells are viable without functional Sp1 protein. For this purpose, embryonic stem cells (ES cells) of the mouse have been used in our laboratory to destroy parts of the genetic sequence that encodes for the Sp1 protein, in a so-called knockout strategy. Surprisingly, these cells can proliferate normally in cell culture. However, mice that lack functional Sp1 protein die at embryonic day 10 *in utero* and show a wide range of abnormalities and malformations. ES cells without functional Sp1 protein are also unable to contribute to embryonic development after this developmental stage when injected into Sp1-proficient host blastocysts.

The work described in this thesis was carried out in order to elucidate some questions arising from the cellular survival, yet embryonic death resulting from Sp1-deficiency.

In **Chapter 2** the search for Sp1 target genes is described carried out with the DNA microarray technology. These experiments have been carried out using ES cells that still carry a functional Sp1 allele by comparing their expression profile to ES cells that do not contain functional Sp1 protein. The rationale behind these experiments was the assumption that Sp1 deficiency leads to changes in transcription of genes that carry recognition sites for Sp1 in their regulatory regions. Indeed, we could detect differences in gene expression, however far fewer genes were affected than could have been expected by the occurrence of Sp1 binding sites in the genome. By Northern blot analysis, so far, cytokeratin19 and stratifin (or 14-3-3σ) could be verified as genes that are less abundantly transcribed in Sp1-deficient ES cells when compared to Sp1-proficient ES cells.

Chapter 3 describes the new finding that ES cells without functional Sp1 are hypersensitive to ionising radiation. Complementation of these cells with a human Sp1 transgene construct resulted in a partial rescue of this phenotype. However, these cells were able to efficiently contribute to embryonic development, when injected into wildtype host blastocysts. The expression of the 14-3-3σ gene is affected by the availability of functional Sp1 (see chapter 2). 14-3-3σ has been described as a key regulator of the check-point at the transition from the G2- to the M-phase of the cell cycle after DNA damage. Therefore, we reckoned that the radiation hypersensitive phenotype of Sp1-deficient ES cells could be due to inefficient expression of 14-3-3σ, which would disable the ES cells to stop the cell cycle at the G2/M transition and gain time to repair the DNA damage induced by ionising radiation. However, forced expression of a 14-3-3σ transgene in Sp1-deficient ES cells could not restore normal sensitivity to ionising radiation. In addition, no gross abnormalities could be detected in the cell cycle responses of Sp1-deficient ES cells when compared to wildtype ES cells. As of now, the reason for the increased sensitivity of Sp1-deficient ES cells towards ionising irradiation is unknown.

The early embryonic death of Sp1-deficient mice precludes the examination of Sp1 functions in later life. Therefore, **Chapter 4** describes the generation of a conditional knockout allele of Sp1 that allows the deletion of Sp1 at later stages in life and in certain organs. For this purpose we applied the Cre/lox system. By use of this system, we flanked the sequence of Sp1 that encodes for the C-terminal part of the protein including the DNA-binding domain by two small sequences called loxP. These small sequences can be recognized by the Cre recombinase from the bacteriophage P1, which is able to remove the sequence between these loxP sites thereby leading to their deletion and the production of a non-functional Sp1 protein. Expression of the Cre recombinase in a tissue-specific manner by use of tissue

specifically active promoters or by activating Cre recombinase at different time-points, allows one to control the place and time of inactivation of the gene of interest.

Two conditional knockout alleles of Sp1 were constructed, differing in the transcriptional direction of the selection cassette compared to that of Sp1. One conditional knockout allele proved to be embryonically lethal in the homozygous state, probably due to transcriptional interference leading to reduced Sp1 expression. Homozygous embryos showed malformations of the head. The second conditional knockout allele generated viable offspring at the homozygous state. These mice were crossed to mouse lines that express the Cre recombinase in different tissues. The cross with a mouse line that expresses Cre in all cells mimicked the phenotype of the conventional Sp1 knock out in that the embryos died *in utero*. However, crossing of conditional Sp1 knock out mice with a mouse line that expresses Cre only in Sertoli cells of the testis and in Schwann cells of the peripheral nervous system resulted in viable mice that were fertile. This implies that Sp1 does not exert vital functions in these cell types. In contrast, a cross of conditional Sp1 knock out mice with a mouse line expressing Cre in developing erythropoietic cells resulted in embryonic lethality at around day 12 of development. This implies that Sp1 exerts a vital function in erythropoiesis at around this time-point of development.

Sp1 conditional knockout mice were also crossed with mice that express an inducible form of Cre, a fusion protein of Cre and a modified form of the ligand binding domain of the estrogen receptor, which should become activated by application of the artificial hormone tamoxifen. However, activation of the Cre recombinase in mice as well as in cells derived from these mice was very inefficient.

In **Chapter 5** we describe the finding that Sp1 heterozygous mice of the C57Bl/6 background develop more eye abnormalities than their wildtype littermates. Using lacZ expression as an indicator of Sp1 expression we show that Sp1 expression can be detected in the lens epithelium and in the neuroretina of the developing eye. As C57Bl/6 mice are prone to eye abnormalities we speculate how reduced Sp1 expression in Sp1 heterozygous mice could lead to the observed increased incidence when compared to wildtype C57Bl/6 mice.

In **Chapter 6** the results and observations of this work are discussed and an outlook on possible future experiments is given.

Samenvatting

De cel is de kleinste eenheid die uit eigen vermogen levensvatbaar is. Deze eigenschap is gecodeerd in het genetisch materiaal van de cel, het DNA. Sommige van de informatie die is opgeslagen in het DNA (genen) wordt via een proces dat transcriptie genoemd wordt overgeschreven in een groep van relatief kortlevende moleculen, de messenger RNA's (mRNA's). De mRNA's zelf worden gebruikt om de uitvoerende moleculen van de cel, de eiwitten, te produceren. Eiwitten hebben veelvoudige functies in de cel. Het zijn bijvoorbeeld bouwstenen voor het skelet van de cel, of sensoren om de voedingsstatus van de cel te bepalen. Eiwitten kunnen ook reacties van de cel op een zich veranderende omgeving uitvoeren. Het geheel van eiwitten in een cel bepaalt de identiteit van een cel. Hersencellen hebben bijvoorbeeld een andere eiwitsamenstelling dan levercellen. Transcriptiefactoren zijn een groep van eiwitten die de productie van andere eiwitten regelen op het niveau van de eerste stap in de eiwitproductie, de transcriptie van het stuk DNA dat voor het eiwit codeert. Dit wordt bereikt door het herkennen en binden aan korte specifieke sequenties op het DNA die te vinden zijn in de regulerende gebieden van genen. Transcriptiefactoren interacteren met het RNA-polymerase, een eiwitcomplex dat de mRNA moleculen genereert, en regelen daarmee de hoeveelheid mRNA moleculen die voor een specifieke eiwit geproduceerd worden.

Dit proefschrift

Hoofdstuk 1 geeft een meer gedetailleerde overzicht van de hierboven genoemde processen en beschrijft de transcriptiefactor Sp1, het onderwerp van dit proefschrift, en aan Sp1 verwante eiwitten.

Sp1 (specificity protein 1) was een van de eerste geïdentificeerde zoogdier transcriptiefactoren. Sp1 is het eerste lid van een familie van transcriptiefactoren die verenigd zijn door het karakteristieke onderdeel waarmee ze aan DNA binden: drie zinc vingers.

Familieleden binden aan specifieke DNA sequenties, zogenoemde GC- of GT boxen, waarmee de belangrijkste basenparen in deze DNA sequenties aangegeven worden. Bindingsplaatsen voor de Sp1 familie van transcriptiefactoren bevinden zich in de regulerende gebieden van talrijke genen.

Omdat Sp1 in allen cellen van het lichaam aanwezig is, was het van belang om te zien of cellen zonder functioneel Sp1 eiwit levensvatbaar zijn. Daarom werden in ons laboratorium in embryonale stamcellen (ES cellen) van de muis DNA sequenties die voor Sp1 codeeren uitgeschakeld met behulp van een zogenoemde knockout benadering. Onverwacht zijn deze cellen in staat om zich verder te vemenigvuldigen. Wel sterven muizen die geen functioneel

Sp1 eiwit kunnen aanmaken als embryo's rond dag 10 van de ontwikkeling. Deze embryo's zijn duidelijk gedeformeerd. ES cellen zonder Sp1 kunnen ook niet meedoen aan de embryonale ontwikkeling na dag 10 als zij in blastocysten (vroege embryo's) geïnjecteerd worden die wel Sp1 kunnen aanmaken.

Het werk dat in dit proefschrift word beschreven werd uitgevoerd om somige vragen te beantwoorden, die werden opgeworpen door de levensvatbaarheid van ES cellen zonder Sp1, in tegenstelling tot de embryonale dood van muizen zonder Sp1.

In **Hoofdstuk 2** wordt beschreven hoe met behulp van de DNA microarray technologie naar target genen van Sp1 werd gezocht. Deze experimenten werden gedaan met ES cellen die nog wel Sp1 kunnen aanmaken in verglijking met ES cellen die dat niet kunnen. Het werd verwacht, dat ES cellen zonder Sp1 een andere expressie patroon van genen laten zien die Sp1 bindingsplaatsen in hun regulerende gebieden hebben. Inderdaad konden wij met deze methode verschillen in genexpressie aantonen, hoewel veel minder dan verwacht gezien de hoeveelheid aan Sp1 bindingsplaatsen in het genoom. Door Northern blot analyse konden wij cytokeratin19 en stratifin (of 14-3-3σ) bevestigen als genen die minder getranscribeerd worden in ES cellen zonder Sp1.

Hoofdstuk 3 beschrijft de tot nu toe onbekende waarneming dat ES cellen zonder Sp1 bijzonder gevoelig voor ioniserende straling zijn. Expressie van een humaan Sp1 transgen in deze cellen kon deze overgevoeligheid gedeeltelijk verminderen, hoewel de transgene ES cellen bij konden dragen aan de embryonale ontwikkeling na injectie in vroege embryo's. Voor het 14-3-3σ gen werd een gereduceerde expressie in ES cellen zonder Sp1 gevonden (zie hoofdstuk 2). 14-3-3σ is in de literatuur beschreven als een gen dat belankrijk is voor de regulatie van de cel cyclus controle punt op de overgang van de G2 naar de M fase van de cel cyclus. Het is daarom mogelijk, dat de overgevoeligheid van ES cellen zonder Sp1 voor ioniserende straling te wijten is aan onvoldoende expressie van 14-3-3σ. Dit zou ertoe kunnen lijden dat de ES cellen na door straling veroorzaakte DNA schade niet kunnen stoppen op de overgang van de G2-naar M fase van de cel cyclus en daardoor geen tijd hebben om de DNA schade te repareren. Expressie van een 14-3-3σ transgen in ES cellen zonder Sp1 kon de overgevoeligheid van deze cellen echter niet opheffen. Bovendien konden we na bestraling geen abnormale cel cyclus in ES cellen zonder Sp1 aantonen. Hierdoor blijft de reden voor de overgevoeligheid van Sp1 knockout ES cellen voor ioniserende bestraling tot nog toe onbekend.

De vroege dood van Sp1 knockout muizen staat onderzoek naar de functies van Sp1 op latere leeftijd in de weg. **Hoofdstuk 4** beschrijft het genereren van muizen waarvan Sp1 op latere leeftijd en alleen in bepaalde organen kan worden uitgeschakeld (conditionele knockout). Daarvoor werd van het Cre/lox-systeem gebruik gemaakt. Met behulp van dit systeem werd de DNA sequentie die voor het C-terminale gedeelte met de DNA-bindende

domeinen van Sp1 codeert, door twee kleine DNA fragmentjes, zogenaamde loxP sites, omgeven. Deze kleine DNA fragmentjes worden door het Cre recombinase van bacteriofaag P1 herkent. Cre kan het DNA stuk dat tussen de twee loxP sites ligt verwijderen. Hierdoor wordt een Sp1 eiwit geproduceerd dat niet functioneel is. Weefsel-specifieke expressie van Cre recombinase met behulp van een weefsel-specifieke promotor, of activatie van Cre recombinase op een bepaalde tijdstip, maken het mogelijk om weefsel en tijdstip van de inactivatie van het Sp1 gen te bepalen.

Twee conditionele knockout constructen van Sp1 werden gegenereerd, waarin de transcriptierichtingen van het selectiegen verschillen. Een van deze constructen bleek in homozygote staat embryonaal letaal te zijn, waarschijnlijk omdat de transcriptie van het selectiegen de transcriptie van Sp1 zodanig stoort, dat er te weinig Sp1 aangemaakt kan worden. Homozygote embryo's hebben misvormingen van het hoofd. Daarentegen zijn muizen met het tweede conditionele knockout construct levensvatbaar in de homozygote staat. Deze muizen werden gekruist met muizen die Cre recombinase in verschillende weefsels tot expressie brengen. De kruising met een lijn die Cre in alle cellen van het lichaam tot expressie brengt bootst het resultaat van de gewone knockout na, omdat de muizen als embryo's doodgaan. Daarentegen bleek het kruisen met een lijn die Cre alleen in Sertoli cellen van de testis en in Schwann cellen van het perifere zenuwstelsel tot expressie brengen, geen effecten te hebben, omdat de muizen levensvatbaar en vruchtbaar zijn. Dit suggereert dat Sp1 geen essentiële functies in deze cellen heeft.

De kruising met een lijn die Cre alleen in zich ontwikkelende rode bloedcellen aanmaakt leidt tot afsterven van de embryo's rond dag 12 van de ontwikkeling. We nemen aan, dat Sp1 een belangrijke functie in de ontwikkeling van rode bloedcellen op deze leeftijd heeft. Sp1 conditionele knockout muizen werden ook gekruist met muizen die een Cre recombinase tot expressie brengen, dat alleen in de aanwezigheid van het kunstmatige hormoon tamoxifen geactiveerd zou moeten worden. We konden slechts een heel inefficiënte activatie van Cre in de muizen, en in gekweekte cellen van deze muizen, waarnemen. Dit induceerbare systeem was daarom niet geschikt voor onze doeleinden.

In **Hoofdstuk 5** wordt beschreven dat Sp1 heterozygote muizen in de C57Bl/6 achtergrond een hogere kans hebben om een abnormale oog ontwikkeling te doorlopen dan wildtype muizen. Door detectie van lacZ expressie als reporter van Sp1 expressie laten we zien, dat Sp1 expressie in het lens epitheel en in de neuroretina van het zich ontwikkelende oog gedetecteerd kan worden. Omdat de normale C57Bl/6 muizen een verhoogde kans op ontwikkeling van abnormale ogen hebben, speculeren we dat de verminderde Sp1 expressie in Sp1 heterozygote muizen deze kans verder verhoogt.

In **Hoofdstuk 6** worden de resultaten en waarnemingen van dit werk bediscussieerd en er wordt een uitzicht op mogelijke verdere experimenten gegeven.

Curriculum vitae

Name	Ulrike Jägle
Born	15 april 1971 in Freiburg/Germany
1981-1990	Grammar school in Müllheim/Germany
10/90 – 3/95	studies in biology at the Albert-Ludwigs-Universität in Freiburg specialisation in genetics, cell biology, biochemistry and physical chemistry practical training in the groups of: Prof. Dr. K.-F. Fischbach at the University in Freiburg and Prof. Dr. A. Preiss at the Biozentrum in Basel/Switzerland working on transgenic Drosophila models PD Dr. C. Bonifer at the University in Freiburg working on transgenic mouse models
3/95 – 3/96	diploma thesis in the laboratory of PD. Dr. C. Bonifer on the "Analysis of the transcriptional activation of chicken lysozyme transgenes during in vitro differentiation of primary murine macrophage precursors"
4/96 – 10/96	temporary scientific staff member in the same laboratory
11/96 – 12/01	Ph.D. studies at the Department of Cell Biology and Genetics, Erasmus University Rotterdam/Netherlands on the "Functional analysis of the transcription factor Sp1"
since 1/02	post-doc position at Novartis company in Basel/Switzerland at the Department of Functional Genomics in the group of Dr. B. Kinzel

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Uli

Stellingen

1. Reduced expression of the transcription factor Sp1 leads to increased incidence of eye abnormalities in a certain mouse strain.
this thesis

2. ES cells without functional Sp1 protein are hypersensitive to ionising radiation.
this thesis

3. Reduced expression of Sp1 leads to increased incidence of abnormal head development during mouse embryogenesis.
this thesis

4. Data of DNA microchip analyses are nowadays obtainable in a reasonable short time period. However, functional validation of the resulting data flood can be very time-consuming.
this thesis

5. Mutant huntingtin protein containing expanded glutamine stretches affects the interaction between Sp1 and its co-activator TAFII130, which leads to inhibition of Sp1-mediated transcription.
(Dunah et al (2002) Science 296: 2238-2243)

6. Cellular oxidative stress in neurons activates Sp1 by enhancing its acetylation, which plays a role in the prevention of oxidative neuronal death.
(Ryu et al (2003) PNAS 100: 4281-4286)

7. Expression of pseudogene RNA can influence the mRNA stability of its protein-encoding homologue.
(Hirotsune et al (2003) Nature 423: 91-96)

8. The transcription factor GATA-1 is essential for the establishment of an erythroid-specific acetylation pattern at the globin gene cluster.
(Letting et al (2003) Mol Cell Biol 23: 1334-1340)

9. In face of the beauty of a mouse embryo, you wonder how people can be interested in anything else than science, until you face the PCR reactions to genotype the embryos.

10. As a German staying in the Netherlands, it is advisable to learn some basic soccer history in order to avoid embarrassing questions.

11. Life is what happens when you've made other plans (John Lennon).

Ulrike Jägle