Studies on biological functions of the transcription factors Sp3 and Sp4

Pieter Fokko van Loo

ISBN: 90-5335-0500

Printed by Ridderprint offsetdrukkerij B.V., Ridderkerk

Studies on biological functions of the transcription factors Sp3 and Sp4

Onderzoek naar biologische functies
van de transcriptiefactoren Sp3 en Sp4

Proefschrift

ter verkrijgen van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus
Prof. dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 13 april 2005 om 15.45 uur

door

Pieter Fokko van Loo

geboren te Hoogeveen.

Promotiecommssie

Promotor:
Prof. dr. F.G. Grosveld

Overige leden:
Prof. dr. D.J.G.M. Duncker
Prof. dr. J.A. Grootegoed
Prof. dr. C.P. Verrijzer

Copromotor:
Dr. J.N.J. Philipsen

Dit proefschrift is tot stand gekomen binnen de Vakgroep Celbiologie aan de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus MC Medische Universiteit Rotterdam. De Vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuid-West Nederland.
Het onderzoek is financieel ondersteund door de Nederlandse Organisatie voor Wetenschappelijk onderzoek NWO.
Het verschijnen van dit proefschrift in boekvorm is mede mogelijk gemaakt door een financiële bijdrage van J.E. Jurriaanse Stichting.

In het begin schiep God de hemel en de aarde.

Genesis 1 vers 1
(nieuwe bijbel vertaling)

Contents

Abbreviations

A	anterior
aaRNA	aminoallyl RNA
Abca8b	ATP-binding cassette, sub-family A 8b
Abdh1	alpha/beta hydrolase domain containing 1
AER	apical ectodermal ridge
AGM	aorta-gonads-mesonephros
Amhc1	atrial myosin heavy chain 1
Ao	aorta
AP2rep	activating protein 2 repressor
Aprt	adenine phosphoribosyl transferase
Bhlh	basic helix-loop-helix
bp	base pair(s)
Bklf	basic klf
Btd-box	Buttonhead-box
Bteb	basic transcription element binding protein
cAMP	cyclic adenosine mono phosphate
cDNA	complementary DNA
CNS	central nervous system
Cnx	connexin
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
Coup TFII	chicken ovalbumin upstream promoter transcription factor II
DC	dendritic cell
Defcr-rs12	defensin related cryptin, related sequence 12
DILV	double inlet left ventricle
DNA	deoxyribonucleic acid
dNTP	deoxy-N-5'-triphosphate
DORV	double outlet right ventricle
DTT	1,4-dithiothreitol
Egf(r)	epidermal growth factor (receptor)
EMP	erythrocyte-megakaryocyte progenitor
Eklf	erythroid klf
EPDC	epicardial derived cell
ErbB	see Egfr
ER	estrogen receptor
ES cell	embryonic stem cell
ET1	endothelin1
Fgf	fibroblast growth factor
Fog1	friend of Gata1
Gklf	gut klf
GMP	granulocyte-monocyte progenitor
GTF	general transcription factor
H2A	histone type 2A
H2B	histone type 2B
H3	histone type 3
H4	histone type 4
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
Iklf	intestine enriched klf
Irx4	iroquois related homeobox 4
Kklf	kidney klf
klf	Krüppel-like factor
L	left
LA	left atrium
Lcn5	lipocalin 5
Lklf	lung klf
LV	left ventricle

mBtd	mouse Buttonhead
mRNA	messenger RNA
Mef2c	myocyte enhancer factor 2c
Mlc2a	myosin light chain 2 atrial
Mlc2v	myosin light chain 2 ventricular
Nhlh2	nescient helix-loop-helix 2
NK cell	natural killer cell
O-GlcNAc	O-linked monosaccharide β-N-acetylglucosamine
P	posterior
PA	pulmonary artery
Pax5	paired box gene 5
Pdgfβ	platelet derived growth factor β chain
PEST	proline, glutamine, serine and threonine enriched sequences
Pka	protein kinase A
Pkcγ	protein kinase C γ
R	right
RA	right atrium
RNA	ribonucleic acid
RNApI	RNA polymerase I
RNApII	RNA polymerase II
RNApIII	RNA polymerase III
rRNA	ribosomal RNA
RV	right ventricle
Rxrα	retinoic x receptor α
SDS	sodium dodecyl sulfate
Slc6a20	solute carrier family 6 member 20
Sp	specificity protein
Spock1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1
Srebp	sterol regulatory element binding protein
SUMO	small ubiquitin-like modifier
SV40	Simian virus 40
T	Brachyury mutation
TAF	TBP-associated factor
TAFII110	TATA-box binding protein-associated factor II 110
TBP	TATA-box binding protein
Tbx5	T-box transcription factor 5
TCRα	T-cell antigen receptor α
TFIIX	transcription factor IIX
Tgfβ	transforming growth factor β
Th1	T helper 1
Th2	T helper 2
Tieg	tgf β inducible early growth response protein
Tpk1	thiamin pyrophosphokinase 1
tRNA	transfer RNA
TSA	histone deacetylase inhibitor trichostatin A
Ttf1	tfhyroid transcription factor 1
Ug/Ccsp	uteroglobin/clara cell secretory protein
U	units
Uklf	ubiquitous klf
UTP	uridine-5'-triphosphate
Vegf	vascular endothelial growth factor
Vmhc1	ventricle specific myosin heavy chain 1
VNO	vomeronasal organ
VSD	ventricular septal defect
XKLF	x Krüppel-like factor
Ypel1	yippee-like 1
Zf9	zinc-finger protein 9
Znf714	zinc-finger protein 741

Scope of this thesis

In this thesis I describe the work that I have executed to study functions of the transcription factors Sp3 and Sp4. Previous experiments on these factors have shown that Sp3 is absolutely essential for normal embryogenesis and that Sp4 is required for post-natal functioning and survival, including sexual behaviour. However, their exact biological roles, and how they fulfil them, remain a mystery. The experiments described in this thesis were designed to come closer to the answers of these questions.

Chapter 1 To place the transcription factors Sp3 and Sp4 in the biological framework of transcription, the first chapter introduces the Sp/XKLF family and in particular the Sp subfamily, and describes the role of transcription factors in the regulation of eukaryotic gene transcription in general, as well as in hematopoiesis and cardiac development.

Chapter 2 Sp3 has been functionally associated with the hematopoietic system. This chapter describes our study that was undertaken to charaterize the role that Sp3 fulfils in the hematopoietic system. This study was performed by an analysis of fetal and adult hematopoiesis in *Sp3*-deficient mice.

Chapter 3 The defect that causes the perinatal death of the $Sp3^{-/-}$ mice remains unknown. As preliminary observations gave a hint that cardiac dysfunction might bring about their perinatal death, we undertook morphological and molecular analysis of $Sp3^{-/-}$ fetal hearts.

Chapter 4 To understand the role of Sp3 in embryogenesis and survival, target genes of Sp3 have to be identified. To identify such genes, we studied and compared the gene expression profiles of fetal $Sp3^{+/+}$ and $Sp3^{-/-}$ hearts by microarray experiments.

Chapter 5 To understand the role of Sp4 in sexual maturation, the impaired sexual behaviour of *Sp4*-deficient males and females was studied in detail by behavioural- and molecular studies.

Chapter 6 Many pieces remain in the puzzle of the biological functions of Sp3 and Sp4. This chapter is a concluding remark on the Sp3 and Sp4 data presented here, and gives an outlook on new experiments that may contribute to elucidate further aspects of the biological roles of Sp3 and Sp4.

CHAPTER 1

Chapter 1

Introduction

Transcription is regulated by transcription factors

Eukaryotic genomes typically contain thousands of genes harbouring information for the proteins that regulate cellular processes. To utilize the encrypted genetic information, genes have to be transcribed into messenger RNA (mRNA), which thereupon is translated into proteins. Transcription of genes into mRNA is highly regulated in time and space to ensure that produced proteins can fulfil their biological role in the development or maintenance of cells and body at the right place and at the right time. In eukaryotes, the transcription of genes into mRNA is performed by the RNA polymerase II (RNApII) enzyme. The two other eukaryotic polymerases, RNApI and RNApIII, transcribe ribosomal RNA (rRNA) and transfer RNA (tRNA), respectively.[1]

The activity of RNApII is regulated by a large protein complex of general transcription factors (GTF), e.g. transcription factor IIB (TFIIB) and TFIID.[2] A representative example of regulation of RNApII during initiation and prolongation of transcription is the interaction of RNApII to promoters containing a TATA-box, an AT-rich sequence located 25-30 bp upstream the transcriptional initiation site. The RNApII is recruited to the TATA-box containing promoter by an association between RNApII and the multi-subunit complex TFIID. A subunit of the TFIID complex, the TATA-box binding protein (TBP), facilitates final binding of the entire protein complex, which includes RNApII, to the TATA-box. Other well known TFIID subunits are the TBP-associated factors (TAFs).[3,4]

However, the basal transcriptional machinery alone is not sufficient to initiate or to direct transcription efficiently. Discovery of sequence-specific DNA-binding proteins or transcription factors revealed that RNApII transcription is directed by cis control elements present in proximal promoter and distal enhancer regions in the genome. Via binding to the cis control elements and via subsequent recruiting of cofactors (corepressors or coactivators) these factors direct the activity of basal transcription machinery and thereby contribute to correct transcription of a gene (review see references [5,6]). Specificity of expression of each individual gene is accomplished by complex arrays of various transcription factor target sequences in the cis control elements of the gene.[7]

DNA in the cell is packaged into chromatin. One of the tasks of transcription factors is to recruit chromatin modifiers to the DNA to modify the condensation grade of chromatin. The first packaging level of chromatin is the wrapping of 146 bp DNA twice around an octamer of histone proteins, composed of two copies of the H2A, H2B, H3, and H4 histones.[8-10] The abovementioned transcription machinery does not have the intrinsic capacity to bind to compacted DNA efficiently.[11] However, some transcription factors can bind their target sequence on packaged DNA and recruit chromatin modifiers to the DNA template to alter the packing grade of the DNA. This modification changes the accessibility of the transcription machinery to the DNA. In this way, these transcription factors contribute to activation or repression of gene expression. One group of chromatin modifiers affects covalent histone modifications (including methylation, acetylation, ubiquitination, and phosphorylation). Another group changes the location and conformation of nucleosomes on the DNA template.[5,12-14]

This brief overview highlights how important sequence specific transcription factors are in directing gene expression. Today, a large number of transcription factors have been identified and most of them have been grouped into families based on similar structural features. One family of transcription factors is the Sp/XKLF (specificity protein/x Krüppel-like factor) family.[15]

The Sp/XKLf family of transcription factors

Introduction to the Sp/XKLF family

Members of the Sp/XKLF family of transcription factors share three conserved Krüppel-like Cys_2Hys_2 zinc-fingers in their C-terminus.[15] This Krüppel-like zinc-finger was first identified in the *Drosophila melanogaster* segmentation Krüppel (Kr) gene.[16] In the late eighties, it was demonstrated that human Sp1, the founder member of the Sp/XKLF family, contains three of these Krüppel-like zinc-fingers.[17] By ongoing research, more factors with three Krüppel-like zinc-fingers were identified in vertebrates. Today, the family contains nine Sp factors (Sp1-Sp9) and 16 KLF factors.[18,19]

The three Krüppel-like zinc-fingers of the Sp/XKLF factors form the DNA-binding domain, which specifically recognizes the GC- (GGGGCGGGG) and GT- (GGTGTGGGG) boxes.[20-23] The boxes are important cis-acting elements in transcriptional activation of housekeeping-, tissue-specific-, cell cycle-, and viral genes as well as for maintenance of the methylation-free status of CpG- islands. This latter feature has been shown for the adenine phosphoribosyl transferase (*Aprt*) gene.[24,25]

The 81 amino acids forming the Krüppel-like zinc-finger of the Sp/XKLF factors are strikingly conserved between all Sp/XKLF factors, including length and amino acid sequence of the spacers between the fingers (see for example Figure 1). This suggests that the entire 81 amino acid stretch is essential for proper functioning of these zinc-finger proteins. From each zinc-finger, three amino acids are thought to contribute to the DNA binding specificity of the Sp/XKLF factors (Figure 1).[26,27] These amino acids are KHA within the first (KLA for Sp2, KHS for Kkl16 and 17), RER within the second, and RHK within the third zinc-finger (RHL for Klf1-8 and Klf12) (Figure 1).[15] The conservation of these critical amino acids between the family members implies that all Sp/XKLF factors recognize the same DNA motifs. However, their affinity for these DNA motifs might differ due to few amino acid alterations in the DNA interacting amino acids or due to amino acid alteration in other parts of the zinc-finger domain that alter the three dimensional conformation of the zinc-fingers.

Sp factors, a subfamily of the Sp/XKLF family

The Sp/XKLF family falls, as the family name already indicates, into two subfamilies: the Sp and the XKLF subfamily.[18] This subdivision within the family is primarily based on common structures that are shared by the Sp factors, and on the unique and remarkable way the Sp genes are localized in the genome. As this thesis discusses the experimental work that

has been performed on the elucidation of the biological function of two Sp factors, namely Sp3 and Sp4, the focus of this overview will be on the Sp subfamily. The overview on the Sp factors commences with a discussion on the common structures and on their chromosomal arrangement in the genome. Next, the transcriptional potential of each Sp factor in relation to their protein composition will be discussed. The overview will continue with a description how post-translational modification modifies their transcriptional potential. Lastly, this overview on the subfamily of Sp factors will outline the functions that the Sp factors execute in vivo.

Sp factors

The founder of this subfamily, Sp1, was discovered as the transcription factor that binds to and activates transcription from multiple GC-boxes in the Simian virus 40 (SV40) early promoter.[2,28] Initially the letters Sp stood for sephacryl- and phosphocellulose, the column used to isolate and purify the Sp1 protein.[17] Nowadays they stand for specificity protein.[15] The initial belief was that Sp1 was the only transcription factor that binds to and acts from all the GC- and GT-boxes. However, subsequent discovery of the closely related Sp2, Sp3, and Sp4 proteins ruled out that belief.[29,30] Recently, another five additional Sp factors have been discovered, namely: Sp5;[31] Sp6 (Klf14 or epiprofin);[32] Sp7 (osterix),[33] Sp8 (mouse Buttonhead or mBtd),[34] and Sp9.[19]

The nine Sp factors share, apart from the zinc-finger domain, several other structural elements. They share the Buttonhead-box (Btd-box) and, with the exception of Sp6, and the

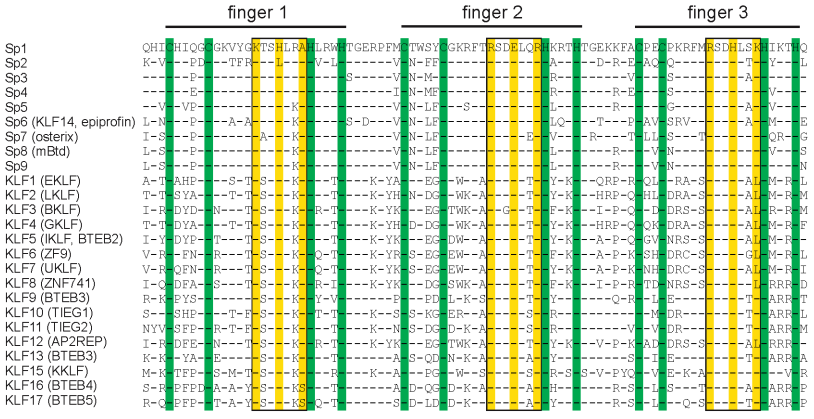


Figure 1. Protein sequence alignment of the 81 amino acid residues forming the Krüppel-like zinc-finger region of the mammalian Sp/XKLF family members
Bars at top highlight the three zinc-fingers of the Sp/Krüppel like factors. The cysteine and histidine residues positioning the Zn-ion in the finger are highlighted in dark grey. In light grey: the amino acids that are thought to contribute to DNA binding specificity of each factor. The amino residues of the fingers that are thought to interact with the DNA bases are boxed. Abbreviations: klf: Krüppel like factor; mBtd: mouse Buttonhead; EKLF: erythroid klf; LKLF: lung klf; BKLF: basic klf; IKLF: intestine enriched klf; UKLF: ubiquitous klf; KKLF: kidney klf; BTEB: basic transcription element binding protein; ZF9: zinc-finger protein 9; ZNF714: zinc-finger protein 741; TIEG: tgf β inducible early growth response protein; AP2REP: activating protein 2 repressor. Depicted are the human sequences, except for Sp9, which is from mouse sequence.

Sp-box (Figure 2). The Btd-box is an eleven amino acid stretch located almost immediately N-terminal to the zinc-finger domain, and was first identified in the *Drosophila* Sp1 homologue Buttonhead (Btd).[31] The Btd-box may be a regulator of transcription activity of the Sp factors, as deletion of domain C in Sp1 (Figure 2) with the Btd-box results in a reduction of *in vitro* Sp1-mediated transcription.[35] In addition, the box may play a role in synergistic activation, as domain C in Sp1 and Sp3 (Figure 2) enhance synergistic activation of sterol regulatory element binding proteins (Srebp) at promoters with a single Srebp binding site.[36,37] However, it is also possible that regulatory characteristics that have been suggested to be a result of the Btd-box are a result of other amino acid stretches within the C-domain. The other domain shared by the Sp factors, the Sp-box is an amino acid sequence SPLALLAATCSR/KI that is present in the N-terminus of the Sp factors (Figure 2).[31] Presence of an endoproteolytic cleavage site in this amino acid stretch suggests that the Sp-box may have a role in regulation of proteolytic cleavage. This idea is emphasized, as the Sp-box in Sp1 partially overlaps with a 54 amino acid sequence that targets Sp1 for proteasome-dependent degradation *in vitro*.[18,38] A second

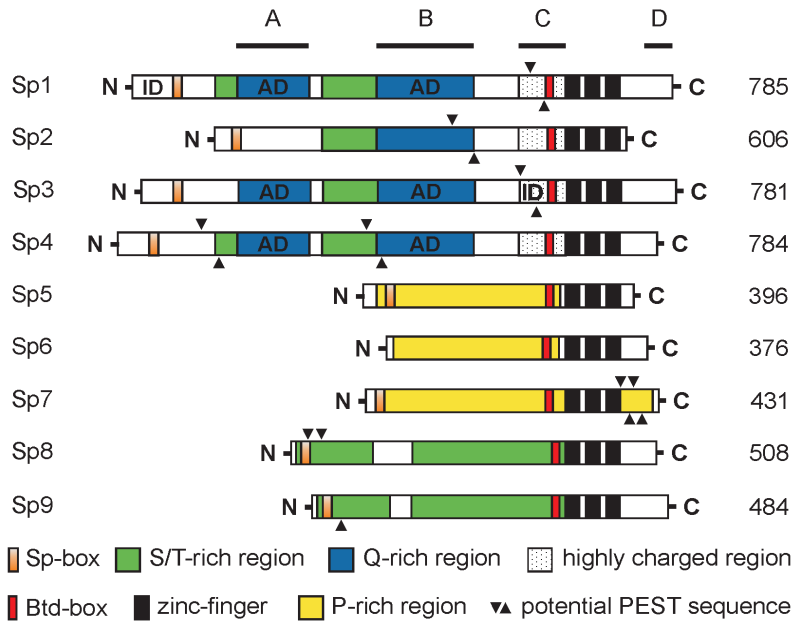


Figure 2. Structural domains of the nine Sp factors
The Sp factors share a number of structural domains in their protein composition. Displayed are the Sp- and Btd-boxes, glutamine-rich, serine/threonine-rich, proline-rich regions, zinc-fingers regions, activations domains (AD), and inhibitory domains (ID). A highly charged region precedes the zinc-finger region of Sp1, Sp2, Sp3, and Sp4. Arrows point at the location of potential PEST domains. Bars at the top indicate the location of the A, B, C, and D domains, initially defined as the regions that contribute to the transcriptional properties of Sp1. These domains have also been assigned to Sp2, Sp3, and Sp4. The protein size in number of amino acids is indicated on the right. If more isoforms are known, size and structural composition of longest isoform is given. Depicted are the compositions of the human protein sequences, except for Sp9, which is from mouse.

function of the Sp-box might lay in control of the transactivation potential via interactions with a putative repressor.[39] Although the functions of both boxes are still poorly understood, their absence in the XKLF subfamily separates the Sp factors from the XKLF factors and proposes them as a subfamily within the Sp/XKLF family, with specific biological functions. An additional shared structural composition by the Sp factors is the PEST (proline, glutamine, serine, and threonine-enriched sequences) domain. Many Sp proteins, but not all, have PEST domains at varying positions (Figure 2, indicated by arrows). Proteins containing a PEST domain can undergo inducible degradation by the 26s proteasome, while they are not ubiquitinated.[40]

Sp1-4 factors form an additional subclass within the Sp subfamily. This subclass is based on similar structural domains. The most striking similarities between these four Sp factors are their glutamine-rich transactivation domain and serine/threonine-rich regions. Sp1, Sp3, and Sp4 have two of these glutamine-rich transactivation domains (domains A and B), accompanied by two serine/threonine-rich stretches at the N-terminal site of both domains (Figure 2).[15] In contrast, Sp2 has only one glutamine-rich transactivation domain with a similarly located serine/threonine-rich domain (Figure 2). All Sp1-4 factors share an additional highly charged C domain, located N-terminal to the zinc-finger region (Figure 2).[15,30]

The nine Sp factors have an unique localization pattern in the human genome. They are all located near a Hox gene cluster, suggesting an expression window similar to the Hox genes, due to sharing of chromatin configuration. *Sp1* and *Sp7* (o*sterix*) co-localize near Hox C on 12q13.13,[41 42] *Sp2* and *Sp6* (*Klf14*) near Hox B on 17q12.31/32,[30,43] *Sp3, Sp5* and *Sp9* near Hox D on 2q31.1,[31,44] and *Sp4* and *Sp8* (mBtd) near Hox A on 7q21.2.[18,45]

Sp factors and their role in transcription

The domains and structures that are shared by the Sp factors are similar, but they are not completely conserved between the eight Sp factors. Furthermore, several Sp factors contain domains that are not present in all of the other Sp factors. All these differences contribute to the unique transcriptional potential of each Sp factor. In this section the focus is on the transcriptional potential of the Sp factors in relation to their domains and boxes, if data is present.

Sp1

Transcription factor Sp1 has strong transcription activation potential that is achieved by a set of structural domains.[35] One of these structures are the zinc-finger domains recognizing GC- and GT-boxes,[29] but also the two glutamine-rich domains (domains A and B) have intrinsic strong transactivation potential and they contribute to the strong transcriptional activation properties of Sp1.[46] Both two glutamine-rich domains are in addition to this essential for superactivation of Sp1-mediated transcription. Superactivation is achieved by interaction of a non-DNA-binding version of Sp1 (the super-activator) with a DNA-binding version of Sp1, resulting in an enhancement of transcriptional activation.[46,47] Although domains A en B are rich in glutamine residues (Figure 2), it is their hydrophobic amino acid residues present

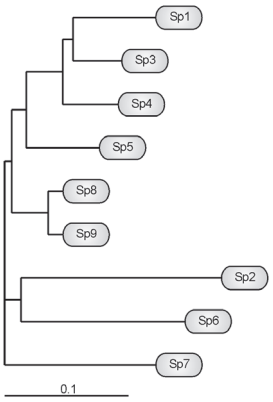


Figure 3. Phylogenetic tree of the Sp transcription factors
For the generation of a phylogenetic tree, the 81 amino acids forming the DNA binding domains of the Sp factors were first aligned with the CLUSTAL W Multiple Sequence Alignment Program (version 1.83).[198] This alignment was subsequently represented in a phylogenetic tree with the Treeview software (version 1.6.6).[199]

in the domains that are essential for the activation properties of domains A and B.[48] The C domain (Figure 2), located N-terminal to the zinc-fingers also has transactivation potential, but it is weak when compared to domains A and B.[46] The most C-terminally located domain of Sp1, domain D (Figure 2), is also involved in the Sp1-Sp1 interaction and thereby facilitates synergistic activation by Sp1 of promoters with adjacent Sp-binding sites. In addition to the Sp1-Sp1 interaction, the D domain is also involved in other protein-protein interactions.[49] Finally, an inhibitory domain has been mapped to the N-terminus of Sp1 (Figure 2),[39] but the regulation of this domain has not been fully characterized yet. Via these structures Sp1 can stimulate transcription from proximal promoters and distal enhancer regions. The Sp1 protein is also able to bring an enhancer region into close proximity of the promoter in an *in vitro* setting. This is achieved by formation of Sp1-tetramers on the proximal promoter as well as the distal enhancer. By subsequent multimerization of these tetramers, the enhancer is brought into close proximity of a promoter[46,49-51] and it is thought that this may promote gene activation. However, Sp1 can also act as a strong transcriptional activator, it has been reported to inhibit Sp3-mediated transactivation of the mouse growth hormone promoter.[52]

An alternative splicing form of the mouse Sp1 lacks domain A and the serine/threonine-rich region of domain B, resulting in a protein with less transcriptional activation potential than full length Sp1 has at similar promoters. Furthermore, this short isoform is inactive at promoters with a single Sp-binding site, but active at promoters with multiple Sp-binding sites. To date, the role of the short Sp1 isoform in transcription mediation has not been elucidated,[53] but it is likely that the short isoform has a specific function in developmental or cellular processes.

Sp2

The Sp2 protein has been identified as a transcription factor that binds weakly to the GT-box in the T-cell antigen receptor α (TCRα) promoter. In contrast to Sp1, Sp2 does not bind to the classical GC-and GT-box sequences, but it binds to a variant of this sequence, namely the 5'-GGGCGGGAC-3' sequence.[30,54] The different binding affinity of Sp2 can be deduced from the relatively large differences in the zinc-finger region of the Sp2 when compared to the corresponding region of the Sp1 protein (Figure 3). The amino acid substitution of a histidine

to a leucine residue in the first zinc-finger of Sp2 is thought to be one of the main differences between the zinc-finger regions of Sp1 and Sp2 that contributes to different binding affinity of the Sp2 protein (Figure 1). In addition to that, Sp2 has a domain at the N-terminal side of the zinc-fingers that was found to regulate the binding capacity of Sp2 to DNA in mammalian cells.[54]

Presence of a N-terminally located glutamine-rich domain postulates Sp2 as a transcriptional activator. However, the transcriptional properties of Sp2 are likely to differ from those of Sp1, Sp3, and Sp4, because it has only one glutamine-rich domain (potentially involved in superactivation) and no D domain (potentially involved in synergistic activation) (Figure 2).[30] This appears to be confirmed by recent experiments showing that Sp2 acts as a weak transcriptional activator.[54]

Sp3

Sp3 is a complicated protein in terms of biological appearance and function. First of all, four isoforms of Sp3 exist. Two of them are large proteins, which migrate as a 110 kD complex (781 en 769 amino acids).[30,55] The other two isoforms are smaller in size, with an approximate size of 70 kD (496 and 479 amino acids).[55-57] All isoforms are translated from four in frame AUG-codons[55] and differ only in the composition of their N-terminus.

The Sp3 proteins share a high degree of structural similarity with Sp1. They have similar zinc-finger domains (Figure 1 and 3), recognizing the GC- and GT-boxes with the same affinity and they have similar N-terminal located glutamine- and serine/threonine-rich domains.[29,30] These glutamine and serine/threonine-rich domains (domains A and B) of Sp3 (Figure 2) have an equivalent transacivation potential equal to that of Sp1.[58,59] However, the two small isoforms lack the most N-terminal located glutamine and serine/threonine-rich domain (domain A), as they are translated form a more downstream AUG codon.

An inhibitory domain is located between the second glutamine-rich domain and the first zinc-finger (Figure 2). This inhibitory domain can turn Sp3 into an inactive transcription factor despite the presence of its N-terminal transactivation domains. The activity of this inhibitory domain is regulated by post-translational modifications. The amino acid sequence IKEE within the inhibitory domain, and especially the lysine residue, is absolutely necessary for this post-translational modification.[58,60-62] The possible post-translational modifications of this inhibitory domain and their effects on the transcriptional activity of Sp3 will be discussed later in this chapter.

In terms of transcriptional mediator Sp3 can act as an activator similar to Sp1.[63-66] However, Sp3 can also act as a very weak activator or even as a transcriptionally inactive protein.[67-69] To understand these at first sight contradictory results, they have to be placed into the context of each individual experiment. In particular, the experiments performed on the transcriptional potential of Sp3 have been carried out with a variety of promoter constructs and in several different cell lines, including *Drosophila* Schneider cells and mammalian cell lines. Thus, the variable results of these experiments suggest that the transcriptional activity of Sp3 is at least dependent on promoter composition as well as cellular context (reviewed in reference [56]). For

example, promoters with a single Sp-binding site are activated by Sp3, whereas promoters with multiple adjacent Sp-binding sites are often not or only weakly activated by Sp3.[58,70] This is caused by efficient competition of Sp3 with Sp1 for binding to promoters containing multiple Sp-binding sites, thus removing the strong transcriptional activator Sp1, resulting in a reduced promoter activity.[58,59,70-72] In addition to its transcriptional activity depending on promoter- and cellular context, Sp3 may also dependent on the distribution of the four isoforms of Sp3. For instance, the long isoforms can activate transcription from the SV40 promoter, whereas the shorter isoforms cannot.[55] However, these long isoforms can also act as weak or inactive proteins, similar to the shorter isoforms.[55,58,70,73]

These data demonstrate that the functional differences between the isoforms cannot solely be explained by the presence or absence of the activation domain A. It is most likely that the structure of the isoforms affects the recruiting of proteins to chromatin, whereas the inhibitory domain regulates the transcriptional potential of the isoforms. The inhibitory domain in turn is regulated by post-translational modifications, which are dependent on the cellular context.

Sp4

The zinc-fingers of the Sp4 protein recognize the GC- and GT-boxes with the same affinity as Sp1 and Sp3.[29,68] The Sp4 protein is also a transcriptional activator due to the presence of two glutamine-rich domains A and B, that are homologous to the glutamine-rich domains of Sp1 (Figure 2). The ability of superactivation of Sp4-mediated transcription by a fingerless Sp1 mutant shows that the glutamine-rich domains of Sp4 are functionally highly related to those of Sp1. However, the function of the D domain of Sp4 (Figure 2) diverges from the function of the Sp1 D domain, as Sp4 is not able to act synergistically via adjacent Sp-binding sites.[74] In addition, the transcriptional activity of Sp4 can be repressed by the Sp3 protein.[68,74]

Sp5

The zinc-finger domain of Sp5 is highly homologous to the corresponding region in Sp1 (Figure 3), especially for amino acids that are thought to interact with the DNA (Figure 1). This results in a similar affinity of Sp5 for the GC-box as Sp1.[31]

Sp6

The Sp6 sequence reveals that the protein lacks the Sp-box and that its N-terminal region, like in Sp5 and Sp7, is rich in proline residues (Figure 2). However, biochemical characteristics of Sp6 have not been studied yet.[32]

Sp7

The DNA-binding domain of Sp7 (Figure 2) binds to the GC-box and other functional G/C-rich sequences. The 27-270 and the smaller proline/serine-rich 27-192 amino acids segments of Sp7 have the ability to act as strong mediators of transcription when fused to a Gal4 binding domain, indicating that Sp7 contains a strong transcriptional activation domain.[33] Two Sp7 isoforms exist in man, created by alternative splicing of the first two exons, and both

are abundantly expressed. As an activation domain is located at the N-terminus of Sp7, the two isoforms may have different transcriptional activation potential.[42]

Sp8

The abundant presence of serine/threonine amino acids in the region upstream the zinc-finger domain set Sp8 apart from the Sp1-7 factors, and postulate Sp8 as a transcription factor that has unique transcriptional potential within the Sp subclass (Figure 2). Regrettably, no transcriptional studies on Sp8 have been reported yet.

Sp9

The Sp9 protein is highly homologous to Sp8 in terms of amino acid composition. Their homology includes the zinc-finger regions (Figure 1 and 3) as well as in the serine/threonine-rich region upstream of the three zinc-fingers (Figure 2).[19] Its biochemical properties, or transcriptional potential have not been reported yet.

Post-translational modifications regulate the activity of Sp factors

Post-translational modifications modulate the biochemical properties of proteins, altering their biological activity. Several Sp factors are modified by one or more post-translational modifications such as glycosylation, phosphorylation, acetylation and SUMOylation. An overview on the known post-translation modifications of the Sp factors will be given.

Glycosylation

Glycosylation modifies serine or threonine residues on cytosolic and nuclear proteins by the O-linked monosaccharide β-*N*-acetylglucosamine (O-GlcNAc).[75,76] Glycosylation affects protein-protein interactions,[77-80] protein stability,[81,82] and subcellular localization.[81] Of the Sp family, Sp1 is known to be glycosylated.[29,30] Sp1 is predominatnly glycosylated via serines and threonine residues located N-terminally in the protein.[75,83] Glycosylation of the glutamine-rich B domain of Sp1 abolishes the interaction of Sp1 with the *Drosophila* TATA-box binding protein-associated factor II 110 (TAFII110). This suggests that glycosylation prevents unauthorized hydrophobic interactions of Sp1 with other proteins,[83] thereby inhibiting Sp1-mediated transcription. This suggestion is confirmed by the observation that Sp1-mediated transcription is inhibited through overexpression of O-GlcNAc transferase.[84] Glycosylation can also increase the stability of Sp1 for proteasome-dependent degradation.[85] This makes Sp1 sensitive to starvation, as glucose starvation combined with adenylate cyclase stimulation leads to hypoglycosylated Sp1 and subsequently increased proteasome-dependent degradation.[82]

Collectively, these data suggest that glycosylation regulates the activity of Sp1 by abolishing unauthorized protein-protein interactions and blocking proteasome-dependent degradation of Sp1. Despite the high degree of homology of Sp3 to Sp1, Sp3 is not found to be glycosylated in vivo. This finding further underlines the conviction that Sp3 is not just a second Sp1 protein.[55] There are no reports on the glycosylation of other Sp factors.

Phosphorylation

Of the Sp subfamily, Sp1 is known to be phosphorylated by a number of kinases at several amino acid residues. The first identified Sp1 kinase is the DNA-dependent protein kinase (DNA-PK) that targets serine residues in the N-terminus of Sp1 and needs DNA-bound Sp1 for efficient Sp1 phosphorylation. This phosphorylation does not affect the DNA-binding capacity of Sp1 or its transcriptional potential. Whether this DNA-PK phosphorylation has other effect on Sp1 is unknown.[86,87] Phosphorylation of Sp1 by cAMP (cyclic adenosine mono phosphate) dependent protein kinase A (PkA) increases its DNA-binding affinity and causes a subsequent transcriptional stimulation of Sp1-dependent promoters.[88] Phosphorylation of Sp1 by protein kinase C γ (Pkcγ) has a similar positive effect on Sp1-mediated transcription.[89] For example, Pkcγ dependent phosphorylation of Sp1 boosts Sp1-mediated expression of several growth factors, such as platelet derived growth factor β chain (Pdgfβ) in smooth muscle cells,[90] and vascular endothelial growth factor (Vegf) in renal carcinomal cells.[89]

Other publications on Sp1 phosphorylation have reported that phosphorylation of Sp1 can reduce the DNA-binding affinity of Sp1, for example in differentiating liver cells[91] or by casein kinase II dependent phosphorylation of a threonine residue in the Sp1 zinc-finger,[92-94] with subsequent reduction in Sp1-mediated transcription.

Phosphorylation of Sp1 has also been linked to degradation. Stimulation of GH_4 cells with epidermal growth factor (Egf) increases Sp1 phosphorylation, resulting in an increased turnover of Sp1 by cysteine proteases.[85]

Reports on phosphorylation of other Sp factors are limited. For Sp3 it has been shown that dephosphorylation, induced by valproic acid, enhances Sp3 binding to DNA probes.[95] Since a number of potential domains for phosphorylation are found in other Sp factors, it is likely that these factors are also targets of this post-translation modification.

Acetylation

As has been mentioned earlier, the activity of the inhibitory domain of Sp3 depends on post-translational modifications. One of these post-translational modifications is acetylation. This domain is acetylated *in vivo,* and it likely that this modification can contributes to the regulation of the transcriptional activity of Sp3. However, the effect of acetylation of the inhibitory domain has not been elucidated.[62]

Recent data from transforming growth factor β receptor II (TgfβRII) promoter analysis suggest that acetylated Sp3 acts a transcriptional activator. Ammanamanchi and co-workers showed that non-acetylated Sp3 represses the TgfβRII promoter region in MCF-7L cells, whereas acetylation of Sp3 by histone deacetylase inhibitor trichostatin A (TSA) treatment, activates this promoter.[96] However, as they did not map the amino acids responsible for this acetylation, it is impossible to compare their data with those of Braun et al., 2001.[62]

SUMOylation of Sp3

The inhibitory domain of Sp3 can undergo a second post-translational modification: SUMOylation. This modification provides another potential mechanism for regulation of the inhibitory domain as well as the activity of Sp3.[60,61] SUMO-proteins are small ubiquitin-like proteins that can be covalently bound to a lysine residue (for review see [97]). Mutational analyses of the inhibitory domain of Sp3 identified the IKXE amino acid sequence as essential for the SUMOylation of Sp3.[60] Sp3 is *in vitro* and *in vivo* SUMOylated by SUMO1 as well as by SUMO2 and this process is mediated by the E3 ligase Pias1 (protein inhibitor of activated Stat1 (signal transducer and activator of transcription 1). SUMOylation of Sp3 silences its activity completely, whereas a Sp3 mutant that cannot be SUMOylated acts as a strong transcriptional activator.[60] In addition, Sp3 is also converted into a strong transcriptional activator in a co-transfection experiment of Sp3 and SUMO1 protease (SuPr1).[61] Silencing of Sp3 upon SUMOylation is not the result of reduced DNA-binding capacity of SUMOylated Sp3, since SUMOylated Sp3 still binds to DNA with normal affinity.[60] Which biological process lies behind silencing upon SUMOylation is not understood yet. Sp3 is SUMOylated through the same lysine residue that can be acetylated.[62] The effect of acetylation on the active of Sp3 is still unknown. A possibly function of acetylation is to block SUMOylation of Sp3, thereby keeping Sp3 as a transcriptional activator.

The *in vivo* function of the Sp factors

The Sp factors share structural domains, including the DNA-binding domain. Despite the similarities in composition, they all have distinct unique biological functions. Unique structural domains and their own expression patterns bring this about. The knowledge on their unique functions comes from studies on the mouse knockouts of the Sp factors. This section will focus on the expression patterns of the Sp factors and on the available knockout phenotypes.

Sp1

Sp1 is ubiquitously expressed[98] and has been assigned to mediate transcription of a large number of genes in many cellular contexts. It has also been implicated in maintenance of methylation-free DNA-islands,[24,25] cell cycle regulation,[70,99-101] and chromatin remodeling.[102-104] Despite that Sp1 might mediate in many basic processes, *Sp1*-deficient embryonic stem (ES) cells are viable and grow normally. In addition, *Sp1*$^{-/-}$ ES cells can differentiate *in vitro* into embryoid bodies.[105] However, *Sp1*-deficient cells cannot survive in the context of developing embryo, since *Sp1*$^{-/-}$ embryos are severely growth retarded compared to wild-type littermates, and die around embryonic day 11 (E11) showing a wide range of abnormalities. Some of these null embryos are nothing more than an undifferentiated mass of cells, while the most advanced embryos develop structures of the heart, limb bud, somites, erythroid cells, and extra-embryonic tissues.[105] The null phenotype indicates that Sp1 is not essential for a particular cell lineage, but for general cellular processes. Injection of

Sp1$^{-/-}$ ES cells into wild-type blastocysts revealed that the *Sp1*-deficient knockout phenotype is cell-autonomous. *Sp1*$^{-/-}$ ES cells give a high contribution to every tissue of early chimeric embryos, but the number of *Sp1*$^{-/-}$ cells contributing to the embryos rapidly declines after E11, and *Sp1*$^{-/-}$ cells are not detected in any tissue of newborn animals.[105]

Sp2

The Sp2 mRNA distribution pattern is supposed to be widely, as several cell lines as well as the rat brain express Sp2.[30,106] Recently, we have generated a *Sp2* knockout mouse and we are currently investigating the physiology of these knockout mice.

Sp3

Despite ubiquitous expression of Sp3, the Sp3-*null* mutant embryos contain all the normal structural elements, such as organs and axial elements. However the null embryos are severely growth retarded compared to wild-type littermates and succumb, shortly after birth. Sp3-null mutant mice in a mixed genetic background of C57Bl/6 and 129/Ola fail to breathe and die within a few minutes after natural delivery or Caesarian section. As they fail to breathe, their lung tissue was examined histologically. *Sp3*$^{-/-}$ lung tissue has smaller alveoli and thicker alveolar septa. The abundance of surfactant proteins A-D and other lung-specific proteins, including thyroid transcription factor 1 (Ttf1) and lung klf (Lklf/Klf2), are normal in *Sp3*$^{-/-}$ lung tissue. The expression of uteroglobin/clara cell secretory protein (Ug/Ccsp) was two-fold decreased in *Sp3*$^{-/-}$ lungs,[107] but *Ug/Ccsp*$^{-/-}$ mice do not suffer from respiratory failure.[108] Thus that lung malformations are not the predominant cause of the perinatal death of the *Sp3*$^{-/-}$ embryos.

Another structure affected in *Sp3*$^{-/-}$ mice is the teeth. The dentin/enamel layer, the layer between the odontoblasts and ameloblasts, has an abnormal morphology in E18.5 Sp3-*null* embryos. This abnormality is reflected by absence of amelogenin and ameloblastin, two ameloblast-specific proteins, in *Sp3*$^{-/-}$ mice.[107] In addition, bone formation is impaired in the *Sp3*$^{-/-}$ mutant. In all parts of the *Sp3*$^{-/-}$ embryos several ossification centers were absent and the expression level of osteocalcin, an osteoblast-specific transcript, is dramatically reduced in *Sp3*-deficient embryos. The reduction in osteocalcin expression indicates that differentiation of preosteoblasts into osteoblasts in *Sp3*$^{-/-}$ mice is delayed or even impaired.[109]

A recently undertaken detailed investigation of the hematopoietic system of *Sp3*$^{-/-}$ embryos revealed that this factor also plays a role in development of this system. The results of this investigation are described in Chapter 2.

Examination of development of the lung as well as the hematopoietic system did not provide an answer to the question what causes the early mortality of Sp3-*null* mice. We observed a high mortality rate of *Sp3*$^{-/-}$ fetuses in the C57Bl/6 background before E18.5, with an increased mortality rate already observed at E16.5. This increased mortality rate, together with the observation of edema formation around E14.5 in *Sp3*$^{-/-}$ fetuses, suggests that Sp3-*null* mice have severely disturbed cardiac development. The detailed investigation of cardiac development in *Sp3*$^{-/-}$ fetuses is described in Chapter 3.

Sp4

The expression of Sp4 is highly dynamic in time and place, with the most prominent in neuronal tissues. Sp4 expression starts around E9 in the posterior neuropore region and later it becomes abundant in developing brain, neural tube, and central nervous system. Additional expression sites are testes, ovaries, and cardiac conduction system. At adult stage, Sp4 is most prominently expressed in brain, skeletal muscle, and epithelial tissues.[29,110-112]

The *in vivo* function of transcription factor Sp4 has been studied in three distinct *Sp4* mutant mouse lines. The first *Sp4* mutant mouse line generated lacks the DNA-binding domain, but still expresses both glutamine-rich activation domains A en B.[111] The second *Sp4* mutant mouse line lacks both glutamine-rich activation domains A and B.[110] The third *Sp4* (*Hf1b*) mutant mouse line has been created by eliminating the Sp4 start codon.[112]

Sp4-deficient embryos are born at a normal Mendelian ratio, but in the first weeks after birth they gain little bodyweight and ~70% die within this period.[110,111] However, this early postnatal death was not observed by Nguyen-Tran et al.[112] This observation suggests that or the genetic background may play a role in early postnatal survival of the $Sp4^{-/-}$ mice, may depend on the knockout strategy that have been used to create the knockout lines. For the surviving $Sp4^{-/-}$ mice, the early reduction in bodyweight is transient. At the adult stage, the $Sp4^{-/-}$ males and females display impaired reproduction. In males this is reflected by the absence of sexual behaviour, despite the apparently normal development of sexual organs and sperm formation. Females have a reduced size of uteri and ovaries, display a delayed onset of puberty, and they rarely mate.[110,111] As Sp4 is abundantly expressed in the brain, the lack of mating behaviour may originate from a brain malformation. However, the vomeronasal organ (VNO) and the hypothalamus, two important brain structures for sexual behaviour, appeared not to be affected by deficiency of Sp4. In addition, two vomeronasal receptors (Vr1-12 and Vr2-9) have a normal distribution in the VNO of newborn $Sp4^{-/-}$ mice.[110] Analysis of the third *Sp4* (*Hf1b*) knockout line revealed that increased sudden cardiac death in the $Sp4^{-/-}$ animals causes the increased postnatal mortality between 6-8 months of age, due to improper functioning of the cardiac conductive system.[112]

Sp5

The Sp5 gene has a dynamic expression pattern throughout early development of the mouse embryo. Its transcript is first detected in the primitive streak. Later on, it is found in the developing brain, spinal cord, branchial arch system, somites, and buds of the limbs. The expression pattern of Sp5 suggests that the protein is involved in many embryonic developmental processes, including gastrulation and organogenesis.[31,113] However, $Sp5^{-/-}$ mice do not display an obvious phenotype, as they are viable and fertile. A link between Sp5 and Brachyury (T) mutation comes from compound mutant mice. Mice carrying the Brachyury (T) mutation on both alleles (*T/T*) have impaired notochord and posterior mesoderm formation, whereas mice heterozygous for the Brachyury mutation (*T/+)* have a shortened and blunted tail.[114,115] This latter phenotype is enhanced in $Sp5^{-/-}$*T/+* compound mutant mice.[31]

Sp6

The primary assignment of the intron-exon distribution of Sp6 (Klf14) was performed by computer analysis. By reverse transcription-polymerase chain reaction, Sp6 was shown to be widely expressed.[116] Nakamura et al. (2004) however revealed the true intron-exon sequence of Sp6 and called the gene epiprofin. By in situ hybridisation this group showed that Sp6 expresses in epithelial cells of teeth, odontoblast, and hair follicles, as well as in the apical ectodermal ridge (AER) of limb buds.[32]

Sp7

Sp7 (o*sterix*) gene has been found in a search for genes encoding osteoblast-specific proteins. Expression of the Sp7 transcript starts at E13 in differentiating chondrocytes and in the surrounding perichondrium. At E15.5 it is additionally expressed in osteoblasts, a cell type that plays a key role in bone formation. Differentiation from preosteoblasts into fully functional osteoblasts is disturbed in the *Sp7*-deficient embryos. This disturbance causes an impaired bone matrix formation in all endochondral and membranous bones, but does not affect cartilage formation. These data indicate that Sp7 enhances preosteoblast differentiation into osteoblasts, and blocks differentiation into chondrocytes (the cartilage forming cells).[33,117]

Sp8

The Sp8 transcript has a distinct expression pattern. It is first expressed in the primitive streak and the neural tube around E8. In later stages, additional Sp8 expression sites are restricted areas of the central neural system, craniofacial regions as well as the AER of the limb buds.[34,118] In Sp8-*null* mutants development of the Sp8-expressing sites is severely affected. *Sp8*$^{-/-}$ mice have an impaired closure of the anterior and posterior neuropore, causing exencephaly, spina bifida, and absence of tail formation.[34] Furthermore, the *Sp8* mutants have severely truncated fore- and hindlimbs, as the AER fails to maintain the expression of early AER markers in the absence of Sp8.[34,118] This phenotype in the AER is caused by the fact that Sp8 positively regulates the expression of fibroblast growth factor 8 (Fgf8),[19,34] which is an essential signalling molecule for limb outgrowth in vertebrates.[119-121]

Sp9

The expression pattern of Sp9 in the mouse partially overlaps with the expression pattern of Sp8, as Sp9 is also expressed in the AER during limb development, the distal region of the ectoderm, and the anterior hindbrain. Experimental work on zebrafish with a mutation affecting pectoral fin formation correlated the Sp9 protein with fin/limb development. The biological function of Sp9 is even more homologous to Sp8, as Fgf10-dependent signalling also regulates the expression of Sp9. However, in contrast to Sp8, expression of Sp9 is not dependent on the Wnt/βcatenin pathway.[19] The function that Sp9 fulfils *in vivo* is rather similar to that of Sp8, as overexpression- or loss of function approaches of Sp9 induce or down-regulate Fgf8, respectively. Similar results were found for Sp8.[19,34] In the absence of a mouse knockout, the function of Sp9 in mammals remains to be determined.

Hematopoiesis and cardiac development

The overview of the phenotypes of the mouse knockouts of the Sp factors shows that Sp factors are involved in many biological processes. Additional research has added new insight on biological functions of Sp factors in hematopoiesis and cardiac development. Therefore, short overviews of hematopoiesis and cardiac development are given. The newly acquired insights on Sp3 and Sp4 will be described in Chapters 3 and 5.

Hematopoiesis

The hematopoietic system provides the organism with a versatile population of hematopoietic cells (Figure 4).[122] The erythrocytes, or red blood cells, are by far the most abundant cell type of the hematopoietic system. These cells have a red colour due to the abundance of hemoglobin molecules that facilitate oxygen supply throughout the body. Furthermore, the erythrocytes remove carbon dioxide from the body by transporting it to the lungs.

In addition to the erythrocytes, the hematopoietic system gives rise to a subset of cells that all fulfil an unique role in maintenance of the vertebrate body. For the initiation of blood clotting in wounded tissues, the hematopoietic system produces platelets. Platelets are not entire cells, but small-detached cell fragments derived from megakaryocytes. Furthermore provides the hematopoietic system the cells of the immune system. These cells, also called white blood cells, bring about an immune response when they encounter a potential pathogen in the vertebrate body. Pathogens are e.g. a microorganism, virus, or a toxic macromolecule that threaten the vertebrate. By an immune response, a pathogen is eliminated, thereby the vertebrate body maintains pathogen-free. The vertebrate immune defence system is subdivided into an innate immune system and an adaptive immune system. The innate immune defence is a protection system of the organism that is immediately available to eliminate a wide range of pathogens, without the requirement of prior exposure. The second immune system, the adaptive immune system, has the capacity to eliminate one specific pathogen from the organism. For such an adaptive immune response this system needs adaptation, which is generated by clonal selection. The adaptive immune system consists of B and T lymphocytes. Each B lymphocyte (or B cell) has an unique receptor on its cell-surface that recognizes a specific pathogen, the antigen. On binding of the pathogen to the receptor, the B cell differentiates into a plasma cell and starts to produce antibodies. An antibody is a secreted and soluble protein that binds to a specific pathogen. This binding neutralizes or prepares the pathogen for destruction by phagocytes. The type of antibody that is produced by the plasma cell has the same pathogen specificity as the cell-surface receptor of the B cell. Of T lymphocytes (or T cells), there are two main classes: the T killer and the T helper class. The first class kills infected cells. Whereas the second class activates other cell types of the immune system, including B cells, to stimulate them to contribute to the elimination of pathogens. Components of the innate immune systems comprehend macrophages, dendritic cells (DCs), mast cells, granulocytes and natural killer cells (NK cells). Macrophages are present in all tissues of an organism and are involved in

phagocytosis and in antigen presentation to the adaptive immune system. They are derived from monocytes, which are cells that circulate in the peripheral blood and continuously differentiate into macrophages upon entering the tissues. DCs are also phagocytic cells, and are presents in the tissues. They leave the blood circulation as immature DC cells, to mature in the tissues into mature DCs that phagocytose pathogens and present pahtogens to T cell, to active the T cells. Like DCs, mast cells also differentiate in the tissues outside the vascular system. Mast cells are believed to play part in protecting mucosal surfaces against pathogens. The granulocytes, also called polymorphonuclear leukocytes, are cells that normally circulate in the blood, but leave the blood circulation to migrate to a site of infection or inflammation. There are three types of granulocytes: neutrophils, eosinophils, and basophils. The neutrophils are involved in phagocytosis, the eosinophils take part in the killing of antibody-coated parasites, and basophils fulfil a function similar to mast cells. The last cell type of the innate immune system, NK cells, are able to recognize and eliminate abnormal cells, such as tumour cells or cells that are infected by viruses. The NK cells circulate in the vascular system, discerning these cells from other type of cells of the innate immune system.

Although mature hematopoietic cells fulfil highly diverse functions in maintenance of the vertebrate body, they all originate from one cell type: the hematopoietic stem cell (HSC) (Figure 4). HSCs are pluripotent cells with intrinsic capacity for self-renewing and to remain active during the entire lifespan of an individual.[123-125] The HSC, which has a very low incidence, can give rise to a stem cell of more limited potential, the hematopoietic progenitor cell (HPC).[126-128] The HPCs are immediate progenitors of the red blood cells, platelets, and the two main categories of the white blood cells (innate and adaptive). Via lineage commitment (Figure 4), these early progenitors develop and differentiate along specific lineages into all mature hematopoietic cells types, as HPCs give rise to the common lymphoid progenitor cell (CLP) and the common myeloid progenitor cell (CMP). The CLPs give rise to the adaptive white blood cells, B and T cells, but also to NK cells of the innate immune system.[129] The CMPs can develop into either the granulocyte-monocyte progenitor (GMP) or the erythrocyte-megakaryocyte progenitor (EMP).[130,131] The GMPs give rise to the three granulocyte classes, the monocytes, and to the as yet elusive precursor of the mast cells. The latter two cell types are immature cells present in the vascular system that mature into, respectively, a macrophage or a mast cell upon migrating into the tissues. The second progenitor that originates from the CMP, the EMP, gives rise to megakaryocytes, of which platelets are derived, and to erythrocytes via the erythoblast intermediate (Figure 4). DCs can, in contrast to other hematopoietic cells, be derived from the CLP as well as the CML. However, as there are more CMPs than CLPs present in the hematopoietic compartments, the majority of the DCs are probably derived from the CMP via the GMP-intermediate.

Hematopoietic cells are produced in distinct phases/waves and anatomical sites.[132-134] The first wave of hematopoiesis, called primitive hematopoiesis, takes place in the blood islands of the yolk sac and starts in the mouse around E7.5.[135,136] These blood islands are derived from the ventral mesoderm layer. Primitive hematopoiesis produces mainly large, nucleated, primitive erythrocytes that contain the embryonic globin proteins.[137,138] The primitive

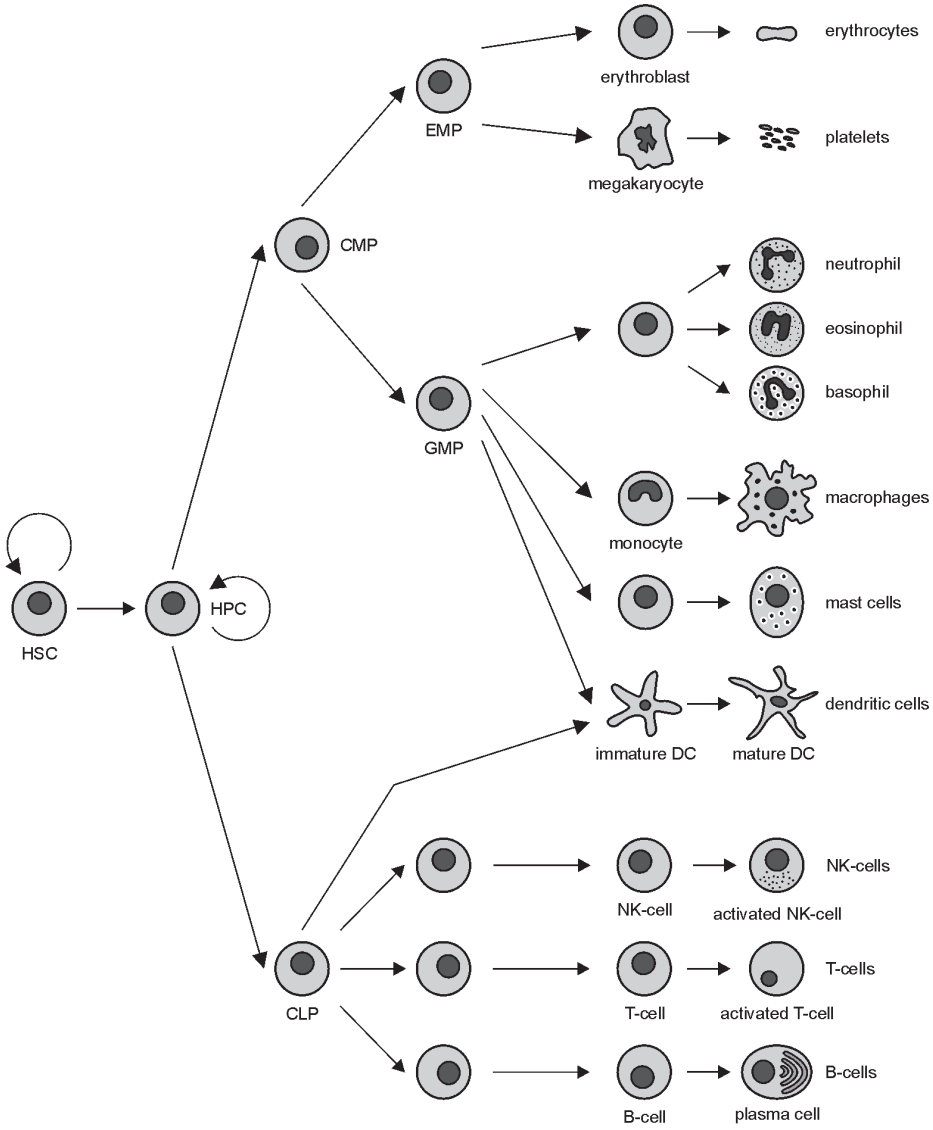


Figure 4. Schematic representation of key stages in the hematopoietic system
The hematopoietic stem cell stands at the basis of the hematopoietic systems and develops via a number of progenitor stages, and along the various hematopoietic lineages into the lineage specific hematopoietic cells. Whereas the hematopoietic stem cell is a pluripotent cell, which possesses the intrinsic capacity for self-renewal, the more downstream progenitors become more and more restricted in their developmental fate and become simultaneously more committed to a specific hematopoietic lineage. HSC: hematopoietic stem cell; HPC: hematopoietic progenitor cell; CMP: common myeloid progenitor; CLC: common lymphoid progenitor; EMP: erythrocyte-megakaryocyte progenitor; GMP: granulocyte-monocyte progenitor; DC: dendritic cell; NK cell: natural killer cell.

erythrocytes leave the blood island to enter the circulation. By that time, they have not gone through their entire developmental program, and they go through a number of cell divisions to finalize their differentiation in the end. The production of erythrocytes by the primitive hematopoiesis stops around E12.5. However, primitive erythrocytes can be detected until E16 in the mouse vascular system.[136]

Primitive hematopoiesis is followed by definitive hematopoiesis. In contrast to primitive hematopoiesis, definitive hematopoiesis gives rise to all hematopoietic lineages found in an adult organism, including enucleated erythrocytes. Definitive hematopoiesis is initiated in the fetal liver around E11 in the mouse.[139] This site of definitive hematopoiesis is temporary, as the location in which definitive hematopoiesis occurs moves during late embryogenesis from the fetal liver to the bone marrow.[140] During late gestation and adulthood, the spleen and thymus are utilized for the differentiation and maturation of lymphoid progenitors. For execution of definitive hematopoiesis, anatomical locations that accommodate this process need to be populated by definitive HSCs to produce all mature hematopoietic cell types (Figure 4). However, the origin of definitive HSCs is an open question, with two divergent models (reviewed by Orkin et al., 2002 [(141)]). The first model says that definitive HSCs originates from the yolk sac, migrating via the aorta-gonads-esonephros (AGM) region (a region formed from the anterior mesoderm), in order to populate the fetal liver.[133,142,143] A second model says that definitive HSCs colonize the liver originate directly from the AGM.[124,144,145]

The differentiation of the HSCs into all blood cell lineages is achieved by sequential commitment of the HSCs to progenitor- and precursors cells with gradually more restricted cell lineage potential. In this process, extracellular and intracellular regulatory molecules are involved. Examples of extracellular effectors are stromal cells in hematopoietic tissue (producing cytokines), growth factors, and cell adhesion molecules. Intracellular general and lineage-specific transcription factors direct the developmental fate of hematopoietic cells. An important family of transcription factors directing proliferation and differentiation of lineage-specific hematopoietic cells is the Gata-family. From this six-member family, only Gata1, -2, and -3 are expressed in the hematopoietic system. Gata1 mediates the survival of erythroid progenitors,[146] the terminal differentiation of erythroid cells, mast cells, megakaryocytes,[147-149] and the specification of eosinophils.[150] Gata2 is essential for maintenance of multipotential hematopoietic progenitors as well as HSCs. Gata2 is also necessary for formation of mast cells.[151,152] Gata3 plays a central role in T-cell development in general, and in regulating T-helper 1 (Th1) and Th2 cell differentiation in particular.[153] Important factors in the development and the specification into lineages are for example friend of Gata 1 (Fog1), PU.1, and paired box gene 5 (Pax5). Fog1 plays an role in erythopoiesis as well as megakaryopoiesis[154,155] and can functionally interact with Gata1.[156,157] PU.1 is involved in granulocytic, monocytic, and lymphoid development.[158,159] Pax5 is important modulator for B-cell development.[160,161]

Members of the Sp/XKLF family are also involved in propagation of hematopoietic progenitors and precursors. The basic Klf (Bklf) factor regulates proliferation of the myeloid lineages[162] whereas the Lklf protein has been implicated in maintenance and viability of mature T cells.[163] In addition, gut Klf (Gklf)[164] and Klf5[165] are associated with T-cell development.

erythroid Klf (Eklf) is essential for the definitive erythroid lineage as mediator of β-globin gene expression.[166,167] Sp1 and EKLF have been reported to interact with Gata1.[168]

Chapter 2 of this thesis shows that transcription factor Sp3 plays a cell-autonomous role in the hematopoietic system and in particular in the erythroid and myeloid cell lineages.

Heart formation

The first organ to function during embryogenesis is the heart. During the whole lifespan of a vertebrate, the heart functions as the pump that circulates the blood through the body. From the onset, the heart undergoes a complicated transformation throughout embryogenesis, directed by highly orchestrated cellular processes. The heart is initially formed as a single linear tube with a single in- and outflow. Via a complex process, this linear tube is remodelled into a heart with two in- and outflow regions and four chambers: two ventricles and two atria. The heart arises from cells of the left and right anterior lateral mesoderm soon after gastrulation. These cells become committed to cardiac fate by signalling products of adjacent dorsal ectoderm, including bone morphogenetic proteins (including Bmp4)[169] and growth factors of the Fgf-family.[170] Thereafter, these cells assemble along the ventral midline of the embryo into a beating linear heart tube, in the mouse around E7.5. The tube is composed of a myocardial layer on the outside and an endocardial layer on the inside, separated by an extracellular matrix (Figure 5a). Next, the linear heart tube undergoes rightward looping, to bring the in- and outflow tracts into close proximity of the four chambers to make proper connections between the heart and the vascular system possible (Figure 5b and 6) (reviewed by Cripps et al., 2002 and Fishman et al., 1997 [(171,172)]). The first cardiac genetic marker expressed by precursor cells is Nkx2-5 (tinman in *Drosophila*). This is one of the most important transcription factors in cardiac development.[173,174] Other early cardiomyocyte-specific genes are members of the myocyte enhancer factor (Mef) and Gata families.[175-177] These factors regulate many cardiac genes during development, often together with Nkx2-5.[178-180]

The looping stage is followed by cardiac chamber specification and septation to form a mature heart (Figure 5c). Important modulators of the ventricular chambers are the two basic helix-loop-helix (bhlh) genes dHand and eHand. dHand is involved in development of the right

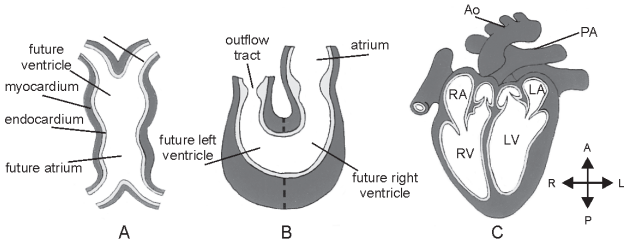


Figure 5. Major stages in development of the vertebrate heart
(a) The heart is initially formed as a linear tube. (b) In time, this linear heart tube undergoes rightward looping, to bring the in- and outflow regions of the heart into close proximity to each other, (c) enabling the formation of a mature four-chambered heart. RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle; PA: pulmonary artery; Ao: aorta; A: anterior; P: posterior; L: left; R: right. (Figure adapted from [200])

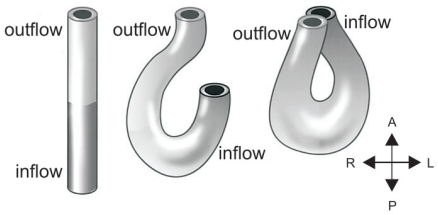


Figure 6. Looping of the heart
The primary heart, composed of a linear tube with a single inflow (posterior) and a single outflow (anterior), undergoes rightward looping to bring the in- and outflow regions into close proximity to each other. This is followed by secondary looping, in which the in- and outflow regions grow towards each other to facilitate formation of a four-chambered heart with double in- and outflow areas. A: anterior; P: posterior; L: left; R: right (Figure adapted from [201])

ventricle[181] whereas eHand is essential in development of the left ventricle.[182] dHand is, together with Nkx2-5, also involved in specification of the heart into ventricles and atria, via regulation of iroquois related homeobox 4 (Irx4).[183] Irx4 stimulates expression of ventricular-specific myosin heavy chain 1 (Vmhc1) and blocks expression of the atrial myosin heavy chain 1 (Amhc1).[184] Atrial growth requires the orphan nuclear receptor Coup TFII (chicken ovalbumin upstream promoter transcription factor II), expressed in atrial precursor cells.[185] T-box 5 (Tbx5) is absolutely essential for atrial and ventricular septation, as Tbx5 deficiency causes severe atrial and ventricular septation defects in mice and humans.[186] Septation of the single cardiac outflow tract, the common truncus, into aorta and pulmonary trunk is achieved by neural crest-derived cells present in the outflow region and condensed mesenchyme.[187-189]

Segmentation of the heart is followed by proliferation of the cardiomyocytes to support the increasing blood flow. Neuregulin growth factors secreted by the endocardium[190] and their ErbB2 (or Egft2, Eepidermal growth factor receptor 2)[191] and ErbB4[192] receptors in the myocardium are required for the formation of trabeculae. The epicardial layer and the epicardial derived cells (EPDCs) are essential for thickening of the compact myocardium.[193,194]

The formation of cardiac valves is absolutely essential for chamber division and for unidirectional blood flow. In the primitive heart, extracellular cardiac cushions (jelly-like structures) achieve unidirectional blood flow. Upon signalling between endocardium and myocardium tissue in the cushion area, mediated by the transforming growth factor β (Tgfβ) pathway, endocardial cells turn into mesenchymal cells, migrate into the cushion and differentiate into fibrous tissue of the valve.[195]

The pulse for contraction of the heart originates from the sino-atrial node and is propagated via the atrium and atrioventricular junction into the ventricles along distal Purkinje fibers. Purkinje fibers are formed from a subset of ventricular cardiomyocytes surrounding the developing coronary artery system, through signalling by endothelin 1 (ET1).[196] The coronary artery system that supplies blood to the heart itself is formed form epicardial-derived cells.

This overview shows that a complex interplay of signalling molecules, receptors, transcription factors and other mediators modulate and direct cardiac development. The Sp/XKLF transcription factors family includes at least two modulators of cardiac development: Sp4 and Lklf. The cardiac phenotype of the *Sp4*$^{-/-}$ mice has already been discussed above. From Lklf it is known that its expression pattern is up-regulated upon shear stress in the heart, and that abnormal shear stress affects cardiac development.[197] Chapter 3 of this thesis describes the crucial role of transcription factor Sp3 in cardiac development, downstream of Nkx2-5.

References

1. Roeder, R. G. & Rutter, W. J. Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature* **224**, 234-7 (1969).
2. Dynan, W. S. & Tjian, R. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* **32**, 669-80 (1983).
3. Tanese, N., Pugh, B. F. & Tjian, R. Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev* **5**, 2212-24 (1991).
4. Dynlacht, B. D., Hoey, T. & Tjian, R. Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* **66**, 563-76 (1991).
5. Kadonaga, J. T. Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell* **116**, 247-57 (2004).
6. Ptashne, M. & Gann, A. Transcriptional activation by recruitment. *Nature* **386**, 569-77 (1997).
7. Dynan, W. S. Modularity in promoters and enhancers. *Cell* **58**, 1-4 (1989).
8. Luger, K., Rechsteiner, T. J., Flaus, A. J., Waye, M. M. & Richmond, T. J. Characterization of nucleosome core particles containing histone proteins made in bacteria. *J Mol Biol* **272**, 301-11 (1997).
9. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**, 251-60 (1997).
10. Thomas, J. O. & Kornberg, R. D. An octamer of histones in chromatin and free in solution. *Proc Natl Acad Sci U S A* **72**, 2626-30 (1975).
11. Imbalzano, A. N., Kwon, H., Green, M. R. & Kingston, R. E. Facilitated binding of TATA-binding protein to nucleosomal DNA. *Nature* **370**, 481-5 (1994).
12. Kadonaga, J. T. Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. *Cell* **92**, 307-13 (1998).
13. Kuo, M. H. & Allis, C. D. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* **20**, 615-26 (1998).
14. Kingston, R. E. & Narlikar, G. J. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev* **13**, 2339-52 (1999).
15. Philipsen, S. & Suske, G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* **27**, 2991-3000 (1999).
16. Schuh, R. et al. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene. *Cell* **47**, 1025-32 (1986).
17. Kadonaga, J. T., Carner, K. R., Masiarz, F. R. & Tjian, R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**, 1079-90 (1987).
18. Bouwman, P. & Philipsen, S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* **195**, 27-38 (2002).
19. Kawakami, Y. et al. Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. *Development* **131**, 4763-74 (2004).
20. Giglioni, B., Comi, P., Ronchi, A., Mantovani, R. & Ottolenghi, S. The same nuclear proteins bind the proximal CACCC box of the human beta-globin promoter and a similar sequence in the enhancer. *Biochem Biophys Res Commun* **164**, 149-55 (1989).
21. Gidoni, D. et al. Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. *Science* **230**, 511-7 (1985).
22. Imataka, K. [Side effects and safety in drug therapy of hypertension]. *Nippon Rinsho* **50 Suppl**, 381-7 (1992).
23. Letovsky, J. & Dynan, W. S. Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. *Nucleic Acids Res* **17**, 2639-53 (1989).
24. Brandeis, M. et al. Sp1 elements protect a CpG island from de novo methylation. *Nature* **371**, 435-8 (1994).
25. Macleod, D., Charlton, J., Mullins, J. & Bird, A. P. Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. *Genes Dev* **8**, 2282-92 (1994).
26. Pavletich, N. P. & Pabo, C. O. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science* **252**, 809-17 (1991).
27. Fairall, L., Schwabe, J. W., Chapman, L., Finch, J. T. & Rhodes, D. The crystal structure of a two zinc-finger peptide reveals an extension to the rules for zinc-finger/DNA recognition. *Nature* **366**, 483-7 (1993).
28. Dynan, W. S. & Tjian, R. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* **35**, 79-87 (1983).
29. Hagen, G., Muller, S., Beato, M. & Suske, G. Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. *Nucleic Acids Res* **20**, 5519-25 (1992).
30. Kingsley, C. & Winoto, A. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. *Mol Cell Biol* **12**, 4251-61 (1992).
31. Harrison, S. M., Houzelstein, D., Dunwoodie, S. L. & Beddington, R. S. Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury. *Dev Biol* **227**, 358-72 (2000).
32. Nakamura, T. et al. The Kruppel-like factor epiprofin is expressed by epithelium of developing teeth, hair follicles, and limb buds and promotes cell proliferation. *J Biol Chem* **279**, 626-34 (2004).

33. Nakashima, K. et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17-29 (2002).
34. Bell, S. M. et al. Sp8 is crucial for limb outgrowth and neuropore closure. *Proc Natl Acad Sci U S A* **100**, 12195-200 (2003).
35. Courey, A. J. & Tjian, R. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* **55**, 887-98 (1988).
36. Athanikar, J. N., Sanchez, H. B. & Osborne, T. F. Promoter selective transcriptional synergy mediated by sterol regulatory element binding protein and Sp1: a critical role for the Btd domain of Sp1. *Mol Cell Biol* **17**, 5193-200 (1997).
37. Yieh, L., Sanchez, H. B. & Osborne, T. F. Domains of transcription factor Sp1 required for synergistic activation with sterol regulatory element binding protein 1 of low density lipoprotein receptor promoter. *Proc Natl Acad Sci U S A* **92**, 6102-6 (1995).
38. Su, K. et al. An N-terminal region of Sp1 targets its proteasome-dependent degradation in vitro. *J Biol Chem* **274**, 15194-202 (1999).
39. Murata, Y., Kim, H. G., Rogers, K. T., Udvadia, A. J. & Horowitz, J. M. Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. *J Biol Chem* **269**, 20674-81 (1994).
40. Rechsteiner, M. & Rogers, S. W. PEST sequences and regulation by proteolysis. *Trends Biochem Sci* **21**, 267-71 (1996).
41. Matera, A. G. & Ward, D. C. Localization of the human Sp1 transcription factor gene to 12q13 by fluorescence in situ hybridization. *Genomics* **17**, 793-4 (1993).
42. Milona, M. A., Gough, J. E. & Edgar, A. J. Expression of alternatively spliced isoforms of human Sp7 in osteoblast- like cells. *BMC Genomics* **4**, 43 (2003).
43. Scohy, S., Van Vooren, P., Szpirer, C. & Szpirer, J. Assignment1 of Sp genes to rat chromosome bands 7q36 (Sp1), 10q31-->q32.1 (Sp2), 3q24-->q31 (Sp3) and 6q33 (Sp4) and of the SP2 gene to human chromosome bands 17q21.3-->q22 by in situ hybridization. *Cytogenet Cell Genet* **81**, 273-4 (1998).
44. Kalff-Suske, M., Kunz, J., Grzeschik, K. H. & Suske, G. Human Sp3 transcriptional regulator gene (SP3) maps to chromosome 2q31. *Genomics* **37**, 410-2 (1996).
45. Kalff-Suske, M., Kunz, J., Grzeschik, K. H. & Suske, G. Human Sp4 transcription factor gene (SP4) maps to chromosome 7p15. *Genomics* **26**, 631-3 (1995).
46. Courey, A. J., Holtzman, D. A., Jackson, S. P. & Tjian, R. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. *Cell* **59**, 827-36 (1989).
47. Pascal, E. & Tjian, R. Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. *Genes Dev* **5**, 1646-56 (1991).
48. Gill, G., Pascal, E., Tseng, Z. H. & Tjian, R. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the Drosophila TFIID complex and mediates transcriptional activation. *Proc Natl Acad Sci U S A* **91**, 192-6 (1994).
49 . Li, R., Knight, J. D., Jackson, S. P., Tjian, R. & Botchan, M. R. Direct interaction between Sp1 and the BPV enhancer E2 protein mediates synergistic activation of transcription. *Cell* **65**, 493-505 (1991).
50. Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P. & Hough, P. V. DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. *Proc Natl Acad Sci U S A* **88**, 5670-4 (1991).
51. Su, W., Jackson, S., Tjian, R. & Echols, H. DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. *Genes Dev* **5**, 820-6 (1991).
52. Yu, J. H., Schwartzbauer, G., Kazlman, A. & Menon, R. K. Role of the Sp family of transcription factors in the ontogeny of growth hormone receptor gene expression. *J Biol Chem* **274**, 34327-36 (1999).
53. Persengiev, S. P., Saffer, J. D. & Kilpatrick, D. L. An alternatively spliced form of the transcription factor Sp1 containing only a single glutamine-rich transactivation domain. *Proc Natl Acad Sci U S A* **92**, 9107-11 (1995).
54. Moorefield, K. S., Fry, S. J. & Horowitz, J. M. Sp2 DNA Binding Activity and trans-Activation Are Negatively Regulated in Mammalian Cells. *J Biol Chem* **279**, 13911-24 (2004).
55. Sapetschnig, A., Koch, F., Rischitor, G., Mennenga, T. & Suske, G. Complexity of translationally controlled transcription factor Sp3 isoform expression. *J Biol Chem* (2004).
56. Suske, G. The Sp-family of transcription factors. *Gene* **238**, 291-300 (1999).
57. Kennett, S. B., Udvadia, A. J. & Horowitz, J. M. Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. *Nucleic Acids Res* **25**, 3110-7 (1997).
58. Dennig, J., Beato, M. & Suske, G. An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. *Embo J* **15**, 5659-67 (1996).
59. Majello, B., De Luca, P. & Lania, L. Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. *J Biol Chem* **272**, 4021-6 (1997).
60. Sapetschnig, A. et al. Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *Embo J* **21**, 5206-15 (2002).
61. Ross, S., Best, J. L., Zon, L. I. & Gill, G. SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell* **10**, 831-42 (2002).
62. Braun, H., Koop, R., Ertmer, A., Nacht, S. & Suske, G. Transcription factor Sp3 is regulated by acetylation. *Nucleic Acids Res* **29**, 4994-5000 (2001).
63. Udvadia, A. J., Templeton, D. J. & Horowitz, J. M. Functional interactions between the retinoblastoma

(Rb) protein and Sp-family members: superactivation by Rb requires amino acids necessary for growth suppression. *Proc Natl Acad Sci U S A* **92**, 3953-7 (1995).
64. Zhao, L. & Chang, L. S. The human POLD1 gene. Identification of an upstream activator sequence, activation by Sp1 and Sp3, and cell cycle regulation. *J Biol Chem* **272**, 4869-82 (1997).
65. Liang, Y., Robinson, D. F., Dennig, J., Suske, G. & Fahl, W. E. Transcriptional regulation of the SIS/PDGF-B gene in human osteosarcoma cells by the Sp family of transcription factors. *J Biol Chem* **271**, 11792-7 (1996).
66. Ihn, H. & Trojanowska, M. Sp3 is a transcriptional activator of the human alpha2(I) collagen gene. *Nucleic Acids Res* **25**, 3712-7 (1997).
67. Majello, B., De Luca, P., Hagen, G., Suske, G. & Lania, L. Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1. *Nucleic Acids Res* **22**, 4914-21 (1994).
68. Hagen, G., Muller, S., Beato, M. & Suske, G. Sp1-mediated transcriptional activation is repressed by Sp3. *Embo J* **13**, 3843-51 (1994).
69. Dennig, J., Hagen, G., Beato, M. & Suske, G. Members of the Sp transcription factor family control transcription from the uteroglobin promoter. *J Biol Chem* **270**, 12737-44 (1995).
70. Birnbaum, M. J. et al. Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. *Biochemistry* **34**, 16503-8 (1995).
71. Yu, B., Datta, P. K. & Bagchi, S. Stability of the Sp3-DNA complex is promoter-specific: Sp3 efficiently competes with Sp1 for binding to promoters containing multiple Sp-sites. *Nucleic Acids Res* **31**, 5368-76 (2003).
72. Zhang, X. et al. Sp1 and Sp3 transcription factors synergistically regulate HGF receptor gene expression in kidney. *Am J Physiol Renal Physiol* **284**, F82-94 (2003).
73. Fandos, C. et al. GLUT1 glucose transporter gene transcription is repressed by Sp3. Evidence for a regulatory role of Sp3 during myogenesis. *J Mol Biol* **294**, 103-19 (1999).
74. Hagen, G., Dennig, J., Preiss, A., Beato, M. & Suske, G. Functional analyses of the transcription factor Sp4 reveal properties distinct from Sp1 and Sp3. *J Biol Chem* **270**, 24989-94 (1995).
75. Jackson, S. P. & Tjian, R. O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. *Cell* **55**, 125-33 (1988).
76. Holt, G. D. & Hart, G. W. The subcellular distribution of terminal N-acetylglucosamine moieties. Localization of a novel protein-saccharide linkage, O-linked GlcNAc. *J Biol Chem* **261**, 8049-57 (1986).
77. Holthofer, H. & Virtanen, I. Glycosylation of developing human glomeruli: lectin binding sites during cell induction and maturation. *J Histochem Cytochem* **35**, 33-7 (1987).
78. Hart, G. W., Haltiwanger, R. S., Holt, G. D. & Kelly, W. G. Glycosylation in the nucleus and cytoplasm. *Annu Rev Biochem* **58**, 841-74 (1989).
79. Hart, G. W. Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. *Annu Rev Biochem* **66**, 315-35 (1997).
80. Hart, G. W., Haltiwanger, R. S., Holt, G. D. & Kelly, W. G. Nucleoplasmic and cytoplasmic glycoproteins. *Ciba Found Symp* **145**, 102-12, discussion 112-8 (1989).
81. Schindler, M., Hogan, M., Miller, R. & DeGaetano, D. A nuclear specific glycoprotein representative of a unique pattern of glycosylation. *J Biol Chem* **262**, 1254-60 (1987).
82. Han, I. & Kudlow, J. E. Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility. *Mol Cell Biol* **17**, 2550-8 (1997).
83. Roos, M. D., Su, K., Baker, J. R. & Kudlow, J. E. O glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions. *Mol Cell Biol* **17**, 6472-80 (1997).
84. Yang, X. et al. O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. *Proc Natl Acad Sci U S A* **98**, 6611-6 (2001).
85. Mortensen, E. R., Marks, P. A., Shiotani, A. & Merchant, J. L. Epidermal growth factor and okadaic acid stimulate Sp1 proteolysis. *J Biol Chem* **272**, 16540-7 (1997).
86. Gottlieb, T. M. & Jackson, S. P. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* **72**, 131-42 (1993).
87. Jackson, S. P., MacDonald, J. J., Lees-Miller, S. & Tjian, R. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell* **63**, 155-65 (1990).
88. Rohlff, C., Ahmad, S., Borellini, F., Lei, J. & Glazer, R. I. Modulation of transcription factor Sp1 by cAMP-dependent protein kinase. *J Biol Chem* **272**, 21137-41 (1997).
89. Pal, S., Claffey, K. P., Cohen, H. T. & Mukhopadhyay, D. Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. *J Biol Chem* **273**, 26277-80 (1998).
90. Rafty, L. A. & Khachigian, L. M. Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta. *Nucleic Acids Res* **29**, 1027-33 (2001).
91. Leggett, R. W., Armstrong, S. A., Barry, D. & Mueller, C. R. Sp1 is phosphorylated and its DNA binding activity down-regulated upon terminal differentiation of the liver. *J Biol Chem* **270**, 25879-84 (1995).
92. Armstrong, S. A., Barry, D. A., Leggett, R. W. & Mueller, C. R. Casein kinase II-mediated phosphorylation of the C terminus of Sp1 decreases its DNA binding activity. *J Biol Chem* **272**, 13489-95 (1997).
93. Ye, X. & Liu, S. F. Lipopolysaccharide down-regulates Sp1 binding activity by promoting Sp1 protein dephosphorylation and degradation. *J Biol Chem* **277**, 31863-70 (2002).
94. Zhu, Q. & Liao, K. Differential expression of the adipocyte amino acid transporter is transactivated by SP1

and SP3 during the 3T3-L1 preadipocyte differentiation process. *Biochem Biophys Res Commun* **271**, 100-6 (2000).
95. Arinze, I. J. & Kawai, Y. Sp family of transcription factors is involved in valproic acid-induced expression of Galphai2. *J Biol Chem* **278**, 17785-91 (2003).
96. Ammanamanchi, S., Freeman, J. W. & Brattain, M. G. Acetylated sp3 is a transcriptional activator. *J Biol Chem* **278**, 35775-80 (2003).
97. Muller, S., Hoege, C., Pyrowolakis, G. & Jentsch, S. SUMO, ubiquitin's mysterious cousin. *Nat Rev Mol Cell Biol* **2**, 202-10 (2001).
98. Saffer, J. D., Jackson, S. P. & Annarella, M. B. Developmental expression of Sp1 in the mouse. *Mol Cell Biol* **11**, 2189-99 (1991).
99. Lin, S. Y. et al. Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Mol Cell Biol* **16**, 1668-75 (1996).
100. Karlseder, J., Rotheneder, H. & Wintersberger, E. Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. *Mol Cell Biol* **16**, 1659-67 (1996).
101. Datta, P. K., Raychaudhuri, P. & Bagchi, S. Association of p107 with Sp1: genetically separable regions of p107 are involved in regulation of E2F- and Sp1-dependent transcription. *Mol Cell Biol* **15**, 5444-52 (1995).
102. Philipsen, S., Pruzina, S. & Grosveld, F. The minimal requirements for activity in transgenic mice of hypersensitive site 3 of the beta globin locus control region. *Embo J* **12**, 1077-85 (1993).
103. Jongstra, J. et al. Induction of altered chromatin structures by simian virus 40 enhancer and promoter elements. *Nature* **307**, 708-14 (1984).
104. Ellis, J. et al. A dominant chromatin-opening activity in 5' hypersensitive site 3 of the human beta-globin locus control region. *Embo J* **15**, 562-8 (1996).
105. Marin, M., Karis, A., Visser, P., Grosveld, F. & Philipsen, S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* **89**, 619-28 (1997).
106. Bigger, C. B., Melnikova, I. N. & Gardner, P. D. Sp1 and Sp3 regulate expression of the neuronal nicotinic acetylcholine receptor beta4 subunit gene. *J Biol Chem* **272**, 25976-82 (1997).
107. Bouwman, P. et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. *Embo J* **19**, 655-61 (2000).
108. Stripp, B. R. et al. Clara cell secretory protein: a determinant of PCB bioaccumulation in mammals. *Am J Physiol* **271**, L656-64 (1996).
109. Gollner, H., Dani, C., Phillips, B., Philipsen, S. & Suske, G. Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**, 77-83 (2001).
110. Gollner, H. et al. Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. *Genes Cells* **6**, 689-97 (2001).
111. Supp, D. M., Witte, D. P., Branford, W. W., Smith, E. P. & Potter, S. S. Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility. *Dev Biol* **176**, 284-99 (1996).
112. Nguyen-Tran, V. T. et al. A novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages. *Cell* **102**, 671-82 (2000).
113. Treichel, D., Becker, M. B. & Gruss, P. The novel transcription factor gene Sp5 exhibits a dynamic and highly restricted expression pattern during mouse embryogenesis. *Mech Dev* **101**, 175-9 (2001).
114. Kispert, A. & Herrmann, B. G. Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. *Dev Biol* **161**, 179-93 (1994).
115. Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. & Lehrach, H. Cloning of the T gene required in mesoderm formation in the mouse. *Nature* **343**, 617-22 (1990).
116. Scohy, S. et al. Identification of KLF13 and KLF14 (SP6), novel members of the SP/XKLF transcription factor family. *Genomics* **70**, 93-101 (2000).
117. Yagi, K. et al. Bone morphogenetic protein-2 enhances osterix gene expression in chondrocytes. *J Cell Biochem* **88**, 1077-83 (2003).
118. Treichel, D., Schock, F., Jackle, H., Gruss, P. & Mansouri, A. mBtd is required to maintain signaling during murine limb development. *Genes Dev* **17**, 2630-5 (2003).
119. Moon, A. M. & Capecchi, M. R. Fgf8 is required for outgrowth and patterning of the limbs. *Nat Genet* **26**, 455-9 (2000).
120. Moon, A. M., Boulet, A. M. & Capecchi, M. R. Normal limb development in conditional mutants of Fgf4. *Development* **127**, 989-96 (2000).
121. Lewandoski, M., Sun, X. & Martin, G. R. Fgf8 signalling from the AER is essential for normal limb development. *Nat Genet* **26**, 460-3 (2000).
122. Metcalf, D. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature* **339**, 27-30 (1989).
123. Lemischka, I. R. The haematopoietic stem cell and its clonal progeny: mechanisms regulating the hierarchy of primitive haematopoietic cells. *Cancer Surv* **15**, 3-18 (1992).
124. Medvinsky, A. & Dzierzak, E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906 (1996).
125. Orkin, S. H. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* **1**, 57-64 (2000).
126. Harrison, D. E., Astle, C. M. & Lerner, C. Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. *Proc Natl Acad Sci U S A* **85**, 822-6 (1988).

127. Harrison, D. E., Jordan, C. T., Zhong, R. K. & Astle, C. M. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Exp Hematol* **21**, 206-19 (1993).
128. Abkowitz, J. L., Golinelli, D., Harrison, D. E. & Guttorp, P. In vivo kinetics of murine hemopoietic stem cells. *Blood* **96**, 3399-405 (2000).
129. Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-72 (1997).
130. Johnson, G. R. & Metcalf, D. Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. *Proc Natl Acad Sci U S A* **74**, 3879-82 (1977).
131. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193-7 (2000).
132. Zon, L. I. Developmental biology of hematopoiesis. *Blood* **86**, 2876-91 (1995).
133. Moore, M. A. & Metcalf, D. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol* **18**, 279-96 (1970).
134. Dzierzak, E., Medvinsky, A. & de Bruijn, M. Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. *Immunol Today* **19**, 228-36 (1998).
135. Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M. & Schulte-Merker, S. The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-66 (1997).
136. Bethlenfalvay, N. C. & Block, M. Fetal erythropoiesis. Maturation in megaloblastic (yolk sac) erythropoiesis in the C 57 B1-6J mouse. *Acta Haematol* **44**, 240-5 (1970).
137. Barker, J. E. Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver. *Dev Biol* **18**, 14-29 (1968).
138. Brotherton, T. W., Chui, D. H., Gauldie, J. & Patterson, M. Hemoglobin ontogeny during normal mouse fetal development. *Proc Natl Acad Sci U S A* **76**, 2853-7 (1979).
139. Ema, H. & Nakauchi, H. Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* **95**, 2284-8 (2000).
140. Bonifer, C., Faust, N., Geiger, H. & Muller, A. M. Developmental changes in the differentiation capacity of haematopoietic stem cells. *Immunol Today* **19**, 236-41 (1998).
141. Orkin, S. H. & Zon, L. I. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nat Immunol* **3**, 323-8 (2002).
142. Palis, J., Robertson, S., Kennedy, M., Wall, C. & Keller, G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-84 (1999).
143. Palis, J. & Yoder, M. C. Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp Hematol* **29**, 927-36 (2001).
144. Medvinsky, A. L., Samoylina, N. L., Muller, A. M. & Dzierzak, E. A. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature* **364**, 64-7 (1993).
145. Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. & Dzierzak, E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301 (1994).
146. Takahashi, S. et al. Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. *J Biol Chem* **272**, 12611-5 (1997).
147. Shivdasani, R. A., Fujiwara, Y., McDevitt, M. A. & Orkin, S. H. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *Embo J* **16**, 3965-73 (1997).
148. Weiss, M. J., Yu, C. & Orkin, S. H. Erythroid-cell-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell line. *Mol Cell Biol* **17**, 1642-51 (1997).
149. Migliaccio, A. R. et al. GATA-1 as a regulator of mast cell differentiation revealed by the phenotype of the GATA-1low mouse mutant. *J Exp Med* **197**, 281-96 (2003).
150. Yu, C. et al. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. *J Exp Med* **195**, 1387-95 (2002).
151. Tsai, F. Y. et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-6 (1994).
152. Tsai, F. Y. & Orkin, S. H. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* **89**, 3636-43 (1997).
153. Farrar, J. D. et al. An instructive component in T helper cell type 2 (Th2) development mediated by GATA-3. *J Exp Med* **193**, 643-50 (2001).
154. Tsang, A. P. et al. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell* **90**, 109-19 (1997).
155. Tsang, A. P., Fujiwara, Y., Hom, D. B. & Orkin, S. H. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev* **12**, 1176-88 (1998).
156. Crispino, J. D., Lodish, M. B., MacKay, J. P. & Orkin, S. H. Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. *Mol Cell* **3**, 219-28 (1999).
157. Fox, A. H. et al. Transcriptional cofactors of the FOG family interact with GATA proteins by means of multiple zinc fingers. *Embo J* **18**, 2812-22 (1999).
158. Scott, E. W., Simon, M. C., Anastasi, J. & Singh, H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265**, 1573-7 (1994).
159. McKercher, S. R. et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities.

Embo J **15**, 5647-58 (1996).
160. Adams, B. et al. Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev* **6**, 1589-607 (1992).
161. Nutt, S. L., Heavey, B., Rolink, A. G. & Busslinger, M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* **401**, 556-62 (1999).
162. Crossley, M. et al. Isolation and characterization of the cDNA encoding BKLF/TEF-2, a major CACCC-box-binding protein in erythroid cells and selected other cells. *Mol Cell Biol* **16**, 1695-705 (1996).
163. Kuo, C. T., Veselits, M. L. & Leiden, J. M. LKLF: A transcriptional regulator of single-positive T cell quiescence and survival. *Science* **277**, 1986-90 (1997).
164. Panigada, M. et al. GKLF in thymus epithelium as a developmentally regulated element of thymocyte-stroma cross-talk. *Mech Dev* **81**, 103-13 (1999).
165. Yang, X. O., Doty, R. T., Hicks, J. S. & Willerford, D. M. Regulation of T-cell receptor D beta 1 promoter by KLF5 through reiterated GC-rich motifs. *Blood* **101**, 4492-9 (2003).
166. Nuez, B., Michalovich, D., Bygrave, A., Ploemacher, R. & Grosveld, F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature* **375**, 316-8 (1995).
167. Perkins, A. C., Sharpe, A. H. & Orkin, S. H. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature* **375**, 318-22 (1995).
168. Merika, M. & Orkin, S. H. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. *Mol Cell Biol* **15**, 2437-47 (1995).
169. Schultheiss, T. M., Xydas, S. & Lassar, A. B. Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121**, 4203-14 (1995).
170. Reifers, F., Walsh, E. C., Leger, S., Stainier, D. Y. & Brand, M. Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar). *Development* **127**, 225-35 (2000).
171. Cripps, R. M. & Olson, E. N. Control of cardiac development by an evolutionarily conserved transcriptional network. *Dev Biol* **246**, 14-28 (2002).
172. Fishman, M. C. & Chien, K. R. Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-117 (1997).
173. Harvey, R. P. NK-2 homeobox genes and heart development. *Dev Biol* **178**, 203-16 (1996).
174. Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I. & Harvey, R. P. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119**, 969 (1993).
175. Lin, Q., Schwarz, J., Bucana, C. & Olson, E. N. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-7 (1997).
176. Gajewski, K., Kim, Y., Choi, C. Y. & Schulz, R. A. Combinatorial control of Drosophila mef2 gene expression in cardiac and somatic muscle cell lineages. *Dev Genes Evol* **208**, 382-92 (1998).
177. Jiang, Y. & Evans, T. The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev Biol* **174**, 258-70 (1996).
178. Sepulveda, J. L. et al. GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol Cell Biol* **18**, 3405-15 (1998).
179. Lee, Y. et al. The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol Cell Biol* **18**, 3120-9 (1998).
180. Durocher, D., Charron, F., Warren, R., Schwartz, R. J. & Nemer, M. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *Embo J* **16**, 5687-96 (1997).
181. Srivastava, D. et al. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat Genet* **16**, 154-60 (1997).
182. Riley, P., Anson-Cartwright, L. & Cross, J. C. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet* **18**, 271-5 (1998).
183. Bruneau, B. G. et al. Cardiac expression of the ventricle-specific homeobox gene Irx4 is modulated by Nkx2-5 and dHand. *Dev Biol* **217**, 266-77 (2000).
184. Bao, Z. Z., Bruneau, B. G., Seidman, J. G., Seidman, C. E. & Cepko, C. L. Regulation of chamber-specific gene expression in the developing heart by Irx4. *Science* **283**, 1161-4 (1999).
185. Pereira, F. A., Qiu, Y., Zhou, G., Tsai, M. J. & Tsai, S. Y. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev* **13**, 1037-49 (1999).
186. Bruneau, B. G. et al. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709-21 (2001).
187. Waldo, K., Miyagawa-Tomita, S., Kumiski, D. & Kirby, M. L. Cardiac neural crest cells provide new insight into septation of the cardiac outflow tract: aortic sac to ventricular septal closure. *Dev Biol* **196**, 129-44 (1998).
188. Poelmann, R. E., Mikawa, T. & Gittenberger-de Groot, A. C. Neural crest cells in outflow tract septation of the embryonic chicken heart: differentiation and apoptosis. *Dev Dyn* **212**, 373-84 (1998).
189. Bergwerff, M., Verberne, M. E., DeRuiter, M. C., Poelmann, R. E. & Gittenberger-de Groot, A. C. Neural crest cell contribution to the developing circulatory system: implications for vascular morphology? *Circ Res* **82**, 221-31 (1998).
190. Meyer, D. & Birchmeier, C. Multiple essential functions of neuregulin in development. *Nature* **378**, 386-90 (1995).
191. Lee, K. F. et al. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-8 (1995).
192. Gassmann, M. et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor.

Nature **378**, 390-4 (1995).
193. Moore, A. W., McInnes, L., Kreidberg, J., Hastie, N. D. & Schedl, A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development* **126**, 1845-57 (1999).
194. Gittenberger-de Groot, A. C., Vrancken Peeters, M. P., Mentink, M. M., Gourdie, R. G. & Poelmann, R. E. Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ Res* **82**, 1043-52 (1998).
195. Brown, C. B., Boyer, A. S., Runyan, R. B. & Barnett, J. V. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science* **283**, 2080-2 (1999).
196. Hyer, J. et al. Induction of Purkinje fiber differentiation by coronary arterialization. *Proc Natl Acad Sci U S A* **96**, 13214-8 (1999).
197. Hogers, B., DeRuiter, M. C., Gittenberger-de Groot, A. C. & Poelmann, R. E. Extraembryonic venous obstructions lead to cardiovascular malformations and can be embryolethal. *Cardiovasc Res* **41**, 87-99 (1999).
198. Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-80 (1994).
199. Page, R. D. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357-8 (1996).
200. Fishman, M. C. & Olson, E. N. Parsing the heart: genetic modules for organ assembly. *Cell* **91**, 153-6 (1997).
201. Kirby, M. L. Molecular embryogenesis of the heart. *Pediatr Dev Pathol* **5**, 516-43 (2002).

Chapter 2

Impaired hematopoiesis in mice lacking the transcription factor Sp3

Pieter Fokko van Loo,[1] Peter Bouwman,[1] Kam-Wing Ling,[1] Sabine Middendorp,[2] Guntram Suske,[3] Frank Grosveld,[1] Elaine Dzierzak,[1] Sjaak Philipsen,[1] and Rudolf W. Hendriks[2]

[1] Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands
[2] Department of Immunology, Erasmus MC, Rotterdam, The Netherlands
[3] Institut für Molekularbiologie und Tumorforschung Philipps-Universität Marburg, Germany

Blood, 1 August 2003, Volume 102, Number 3, pages 858-866

CHAPTER 2

Abstract

As the zinc-finger transcription factor specificity protein 3 (Sp3) has been implicated in the regulation of many hematopoietic-specific genes, we analysed the role of Sp3 in hematopoiesis. At embryonic day 18.5 (E18.5), *Sp3*$^{-/-}$ mice exhibit a partial arrest of T-cell development in the thymus and B-cell numbers are reduced in liver and spleen. However, pre-B-cell proliferation and differentiation into immunoglobulin M-positive (IgM^{+})B cells in vitro are not affected. At E14.5 and E16.5, *Sp3*$^{-/-}$ mice exhibit a significant delay in the appearance of definitive erythrocytes in the blood, paralleled by a defect in the progression of differentiation of definitive erythroid cells in vitro. Perinatal death of the *null* mutants precludes the analysis of adult hematopoiesis in *Sp3*$^{-/-}$ mice. We therefore investigated the ability of E12.5 *Sp3*$^{-/-}$ liver cells to contribute to the hematopoietic compartment in an in vivo transplantation assay. *Sp3*$^{-/-}$ cells were able to repopulate the B- and T-lymphoid compartment, albeit with reduced efficiency. In contrast, *Sp3*$^{-/-}$ cells showed no significant engraftment in the erythroid and myeloid lineages. Thus, the absence of Sp3 results in cell-autonomous hematopoietic defects, affecting in particular the erythroid and myeloid cell lineages.

Introduction

The transcription factor specificity protein 3 (Sp3) belongs to the Sp/X Krüppel-like factor (XKLF) family of nuclear proteins that share a zinc-finger domain containing 3 C_2H_2-type zinc fingers related to those found in the *Drosophila melanogaster* regulator protein Krüppel.[1] Sp1, Sp3, and Sp4 bind with equal affinity to the classical GC box and the related GT/CACC boxes present in many housekeeping and tissue-specific genes. Sp1 and Sp3 are widely expressed, whereas Sp4 shows a complex expression pattern but is most abundant in neuronal tissues. Sp factors not only are able to stimulate transcription from proximal promoters or from distal enhancers,[2] but can also physically interact with other transcription factors, such as E2F-I, nuclear factor-κB (NF-κB), and GATA-1.[3-5] Sp1 and Sp3 can have additive or synergistic effects on gene activation, but Sp3 is also able to repress transcription driven by Sp1 or other transcription factors.[6,7]

The characterization of mutant mice has provided important information about the biologic function of the individual Sp/XKLF proteins. Yet only a limited number of target genes for the Sp/XKLF family members have been identified to date. In particular, the G-rich sequences in the locus control region (LCR) of the β-globin cluster were identified as direct targets of Sp/XKLF transcription factors. Sp1, Sp3, basic KLF (BKLF), and erythroid KLF (EKLF) are all present in erythroid cells, but only EKLF appears to be essential for β-globin expression and activation of the LCR.[8-10] EKLF-deficient mice die in utero owing to a severe anemia caused by severely reduced β-globin expression.[8,9] BKLF has a role in myeloid proliferation, as *BKLF*$^{-/-}$ mice have increased numbers of myeloid cells, which also show increased proliferation rates in vitro.[11] Lung KLF (LKLF) is required for blood vessel development and has been implicated in the maintenance of mature T cells.[12,13] Sp1-*null* embryos are severely retarded in growth and die after day 10 of embryonic development (E10). As *Sp1*$^{-/-}$ cells did not contribute to any tissue of newborn chimeric animals, the Sp1 deficiency causes a cell-autonomous defect.[14] *Sp3*$^{-/-}$ embryos are also growth retarded, resulting in prenatal lethality or death at birth, apparently owing to respiratory failure.[15] The cause for the observed breathing defect remains obscure, as only minor morphologic alterations were observed in the lung and surfactant protein expression was normal. Furthermore, *Sp3*$^{-/-}$ mice show a pronounced defect in late tooth formation associated with a deficiency of ameloblast-specific transcripts,[15] and impaired skeletal ossification, reflected by significantly diminished expression of the osteoblast-specific marker gene osteocalcin.[16] The phenotype of *Sp4*$^{-/-}$ mice differs from those described for *Sp1*$^{-/-}$ and *Sp3*$^{-/-}$ mice, as they develop until birth without obvious abnormalities. After birth, two thirds of the knockout mice die within 4 weeks as a consequence of cardiac arythmia.[17] Surviving mice are growth retarded, and male *Sp4*$^{-/-}$ mice do not breed.[18]

Sp3 was originally cloned in a search for Sp1-related factors that bind to a GT box required for the transactivation of a T-cell receptor (TCR) V_α gene segment.[19] Since then, numerous functional binding sites for Sp factors have been identified that implicate Sp factors in the regulation of T- and B-cell development, for example, in TCR and immunoglobulin-enhancer

regions, the promoter that directs germ line transcription of DβJβ gene segments in precursor T lymphocytes, the interleukin-2 receptor (IL-2R) β-chain, and promoter regions of genes encoding the B-cell receptor signaling molecules immunoglobulin-α (Ig-α), Ig-β, and Bruton tyrosine kinase (Btk).[2,19-25] In addition, Sp1/Sp3 sites have been shown to be involved in the regulation of macrophage colony-stimulating factor (M-CSF), which is essential for myeloid development.[26]

Therefore, the presence of Sp-binding sites in various regulatory regions of lymphoid-, erythroid-, and myeloid-specific genes prompted us to investigate hematopoietic development in Sp3-deficient mice. As the *Sp3* gene was disrupted by targeted insertion of a *lacZ* reporter gene,[15] we could quantify the Sp3 expression profile in the individual hematopoietic compartments by analysis of β-galactosidase activity. When we compared $Sp3^{-/-}$ embryos with wild-type littermates, we identified a significant delay in the formation of T and B lymphocytes at E18.5, and erythrocytes at E14.5 and E16.5. Moreover, Sp3-deficient E12.5 liver cells did not significantly repopulate the erythroid or myeloid cell lineages in an in vivo transplantation assay. Our findings indicate that the lack of Sp3 intrinsically affects hematopoietic development, especially of the erythroid and myeloid lineages.

Results

Expression of the *lacZ* knock-in reporter gene during T-cell development in the thymus

As a role for Sp3 in particular hematopoietic cell lineages may be indicated by modulations of the *Sp3* gene expression, we took advantage of the presence of a *lacZ* reporter gene in the targeted allele of $Sp3^{+/-}$ heterozygous mice, which was placed under direct Sp3 transcriptional control[15] (Table 1; Figure 1). The expression of Sp3-directed *lacZ* was analyzed in flow cytometry experiments, with the use of FDG as a fluorogenic β-galactosidase substrate in conjunction with antibodies specific for the individual hematopoietic cell lineages.

In E18.5 $Sp3^{+/-}$ embryos, we found high levels of *lacZ* activity in the B-lymphoid and myeloid compartments. The differentiation of large into small $Ter119^{+}$ erythroid cells was associated with a decrease of *lacZ* activity (Table 1). In the thymus, we identified a modulated expression profile, characterized by a significant downregulation of *lacZ* expression when differentiating T cells started to express the TCR/CD3 complex (Figure 1a). In the most immature population of $CD3^{-}CD4^{-}CD8^{-}$ triple-negative (TN) thymocytes, *lacZ* expression was detected in more than 80% of the cells. Also the 4 subpopulations of these TN cells, as defined by differential CD44 and CD25 expression invariably showed greater than 80% $lacZ^{+}$ cells (data not shown). High levels of *lacZ* activity were maintained in the next stages of T-cell development, that is, the $CD3^{-}CD8^{+}$ immature single-positive (ISP) cells, which have successfully rearranged their TCRβ locus, and the $CD3^{-}CD4^{+}CD8^{+}$ double-positive (DP) cells, which are in the process of TCRα gene rearrangement (Figure 1b). After successful TCRα rearrangement, a complete TCRαβ is

expressed on the cell surface, and subsequently a relatively small number of TCRαβ-bearing DP cells are selected for major histocompatibility complex (MHC) recognition and up-regulate surface expression of the TCRαβ/CD3 complex during the process of positive selection.[36] At this stage, $CD3^+$ DP cells down-regulate surface CD4/CD8 coreceptor expression, to become $CD4^{low}CD8^{low}$ double-dull (DD) cells.[37] Remarkably, at the $CD3^+CD4^+CD8^+$ DP and DD cell stages, the proportion of *lacZ*-expressing cells was significantly down-regulated to approximately 20%. When cells further differentiated into mature CD4 single-positive (SP) cells, the proportion of $lacZ^+$ cells increased to approximately 50%. At E18.5, CD8 single-positive cells were not yet detectable in the thymus.

In summary, these analyses indicate that Sp3 is abundantly expressed in the various hematopoietic lineages. In contrast, during T-cell development, *lacZ* expression is high in early $CD3^-$ precursor T-cell stages, specifically down-regulated during positive selection of $TCR\alpha\beta^+$ DP T cells, and finally up-regulated in CD4 SP cells.

Partial arrest of T-cell development at the DP stage in *Sp3*-deficient embryos

At E18.5, $Sp3^{-/-}$ embryos are smaller (approximately 75%) than their wild-type littermates.[15] With this taken into consideration, the development of the thymus appeared to be relatively more affected, as $Sp3^{-/-}$ E18.5 embryos had a considerably smaller thymus, containing only approximately 30% of the cell numbers of wild-type littermates (Figure 2a). To analyze the effect of Sp3 expression on T-cell development in more detail, we determined the sizes of the individual T-cell precursor subpopulations in the thymus of $Sp3^{-/-}$, $Sp3^{+/-}$, and $Sp3^{+/+}$ mice at E18.5 by flow cytometry (Figure 2b). These analyses revealed that in $Sp3^{+/-}$ embryos, the various T-cell precursor populations were present in near-normal abundance, except for the CD4 SP population, which was approximately 60% of normal size. In contrast, the absence of Sp3 resulted in a partial arrest of T-cell development, as the numbers of DP and CD4 SP cells

Table 1. Expression of *lacZ* in hematopoietic lineages in $Sp3^{+/-}$ E18.5 embryos

Organ and cell population	Fraction of *lacZ* cells, %
Liver	
$B220^+$ B-lineage cells	92 ± 1.4
$Ter119^+$ erythroid, large FSC	75 ± 2.4
$Ter119^+$ erythroid, small FSC	45 ± 3.4
Spleen	
IgM^-B220^+ pre-B cells	93 ± 0.7
IgM^+B220^+ B cells	92 ± 3.7
$Ter119^+$ erythroid, large FSC	90 ± 2.6
$Ter119^+$ erythroid, small FSC	38 ± 3.7
Bone marrow	
$Ly\text{-}6C^{med}$ granulocyte precursors*	72 ± 3.8
$Ly\text{-}6C^{high}$ monocyte precursors	89 ± 1.1

Data are presented as mean values ± standard deviation (SD); n = 4
FSC indicates forward scatter
* See de Bruijn et al.[28]

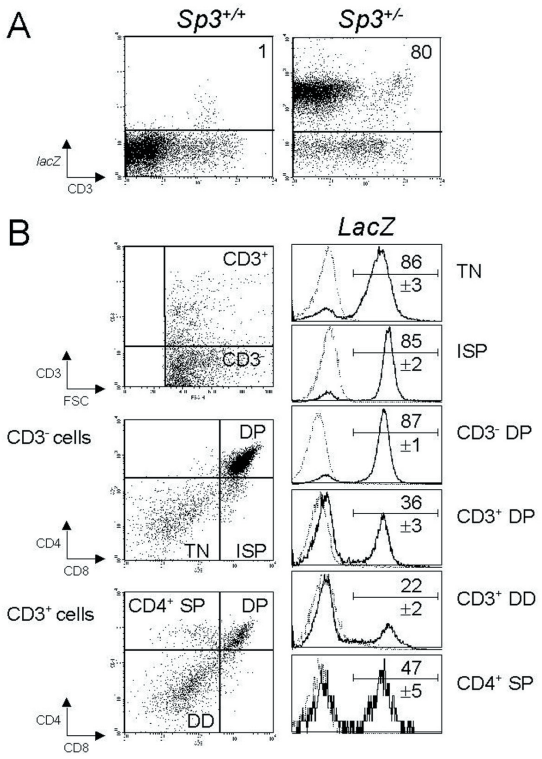


Figure 1. Analysis of *lacZ* expression in thymocytes from *Sp3*$^{+/-}$ embryos by 4-color cytometry

Thymus cell suspensions were loaded with FDG substrate and subseqently stained for CD3, CD4, and CD8. (a) Total thymocytes were analyzed for CD3 expression and *lacZ* activity. (b) Cells were analyzed for CD3 expression; CD3$^-$ and CD3$^+$ fractions were analyzed for CD4 and CD8; and the indicated subpopulations were gated and analyzed for *lacZ* activity. The results are displayed as histograms of *Sp3*$^{+/-}$ embryos (bold lines), together with those of wild-type embryos (dashed lines). The numbers are the proportions of *lacZ*-expressing cells (mean values ± SD, n = 6). Background values in wild-type mice (n = 4) were below 1% in all fractions, except for the CD3$^+$ double-dull (DD) fraction, in which 3% ± 1% of the cells were *lacZ*$^+$.

in the *Sp3*$^{-/-}$ mice were reduced by a factor greater than 3 and greater than 7, respectively, when compared with wild-type littermates.

As these findings indicate that Sp3 deficiency affects T-cell development, particularly at the DP stage, we investigated *Sp3*$^{-/-}$ and *Sp3*$^{+/+}$ DP thymocytes in more detail. We observed that *Sp3*$^{-/-}$ CD3$^-$ DP cells had increased average forward scatter values, suggesting that a large proportion of the Sp3-deficient DP cells still have morphologic characteristics of the actively cycling ISP cells (Figure 2c). However, an increased proliferation rate of *Sp3*$^{-/-}$ cells at the DP cells seems unlikely, as the total size of the DP compartment in *Sp3*$^{-/-}$ mice is decreased. Rather, it suggests that the Sp3-deficient DP cells still bear hallmarks of the previous stage owing to a defect in developmental progression. It has been shown that upon MHC/TCRαβ interaction, DP develop into DD cells, up-regulate expression of the TCR/CD3 complex, and start to express the very early activation antigen CD69 on the membrane.[37] When we specifically analyzed DP and CD4^{low}CD8^{low} DD cells, we found that in the absence of Sp3 the induction of CD3 and CD69 expression on the surface of DD cells was significantly reduced (Figure 2d).

In summary, we conclude that the *Sp3*$^{+/-}$ and *Sp3*$^{-/-}$ embryos manifest a defect in thymocyte maturation at the DP cell stage. This arrest in differentiation coincides with the observed downregulation of *lacZ* expression during positive selection of TCRαβ$^+$ DP T cells.

Impaired B-cell development in *Sp3*-deficient embryos

The absence of Sp3 also affected the B-cell system. E18.5 *Sp3*$^{-/-}$ livers were smaller in size and manifested a reduction of both total cellularity and the absolute numbers of B220^{+} B-lineage cells, when compared with livers from wild-type littermates (Figure 3a). Moreover, detailed flow cytometric analysis ofliver and spleen demonstrated an approximately 2-fold lower percentage of surface IgM^{-} B-cell precursors and an approximately 5-fold lower percentage of IgM^{+} B cells than normally found at this age (Figure 3b). Intermediate values were found for *Sp3*$^{+/-}$ mice, indicating an *Sp3* gene dosage effect.

To investigate whether Sp3-deficient pre-B cells have an intrinsic defect in proliferative expansion or differentiation into Ig^{+} B cells, we performed in vitro IL-7-driven fetal liver pre-B-cell cultures. E18.5 fetal liver cells were cultured in the presence of 100 U/mL IL-7, thereby specifically inducing proliferation of cytoplasmic Igμ heavy-chain-positive pre-B cells.[29] After 5 days of culturing, comparable numbers of B220^{+}IgM^{-} pre-B cells were generated in *Sp3*$^{+/+}$, *Sp3*$^{+/-}$, and *Sp3*$^{-/-}$ fetal liver cultures. Subsequently, IL-7 was removed from the medium, and the cells were placed on S17 stromal cells for 48 hours to allow further differentiation. Flow cytometric analysis demonstrated that *Sp3*$^{-/-}$ pre-B cells differentiatednormally into surface IgM^{+} immature B cells upon IL-7 withdrawal. Furthermore, *Sp3*$^{-/-}$ B cells were able to differentiate into mature IgM^{+}IgD^{+} B cells, although

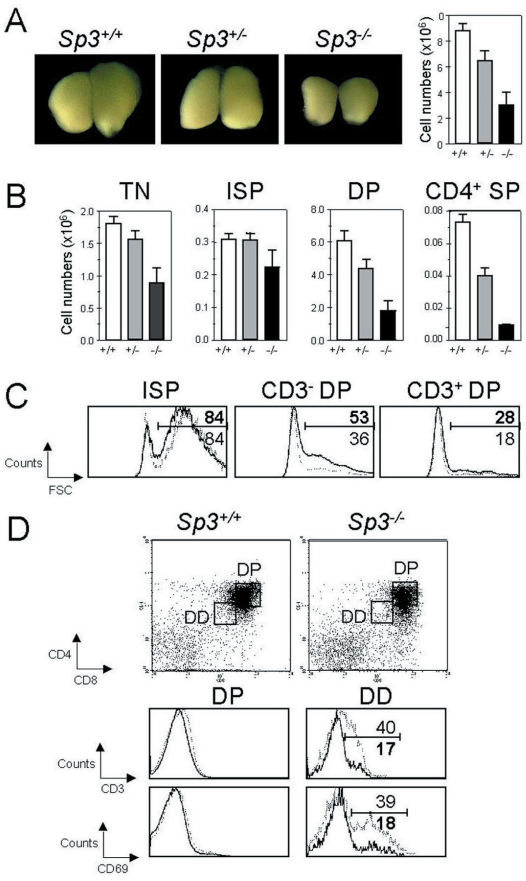


Figure 2. Impaired T-cell development in embryos lacking Sp3
(a) Thymi of E18.5 embryos of one litter (left) and total numbers of thymocytes (right) of the indicated genotypes. (b) Absolute numbers of thymocytes within the indicated thymic subpopulations, as determined by flow cytometry. Tn = CD3^{-}CD4^{-}CD8^{-}; ISP = CD3^{-}CD8^{+};DP = CD4^{+}CD8^{+}; and CD4 SP = CD3^{+}CD4^{+}CD8^{-}. (c) FSC characteristics of the indicated thymus subpopulations. Cells were analyzed for the expression of CD3, CD4, and CD8, and FSC characteristics are displayed as histogram overlays of *Sp3*$^{-/-}$ (bold lines) and *Sp3*$^{+/+}$ (dashed lines) embyros. (d) CD3 and CD69 expression in DP (CD4^{+}CD8^{+}) and DD (CD4^{low}CD8^{low}) thymocytes. Thymus cell suspensions were gated for CD4 and CD8, and results are displayed as histogram overlays for CD3 and CD69 of *Sp3*$^{-/-}$ (bold lines) and *Sp3*$^{+/+}$ (dashed lines) embyros. Data shown are from 2 to 26 embryos in each group.

apparently with a reduced efficiency, as lower levels of surface IgD expression were observed in *Sp3*$^{-/-}$ cultures (Figure 3c).

Taken together, these results indicate that in the absence of Sp3, B-cell development was impaired, as the numbers of (pre-)B cells in the liver and spleen of *Sp3*$^{-/-}$ embryos did not reach the levels of normal embryos. The IL-7-driven fetal liver culture experiments show that the proliferative capacity of *Sp3*$^{-/-}$ pre-B cells and their developmental progression into IgM^{+} immature B cells in vitro are normal.

The formation of definitive erythrocytes is delayed in *Sp3*-deficient embryos

To study the effect of the absence of Sp3 on erythropoiesis, the number and the size of erythrocytes were analyzed in E12.5, E14.5, and E16.5 embryos (Figure 4a). Blood of wild-type embryos contained predominantly large primitive erythroid cells at E12.5, mainly small definitive erythrocytes at E14.5, and almost exclusively small definitive erythrocytes at E16.5, as described previously.[38] In contrast, in E14.5 *Sp3*$^{-/-}$ blood, the majority of red cells were large primitive nucleated erythrocytes. A significant fraction of these primitive erythrocytes was still present at E16.5. As shown in Figure 4a, a delay in the formation of small definitive erythrocytes was also found in *Sp3*$^{+/-}$ blood, but the phenotype was much less striking. These findings were confirmed in cytospin analyses, where, particularly at E14.5, increased proportions of nucleated primitive erythroid cells were detected in the blood from *Sp3*$^{-/-}$ embryos and, to a lesser extent, also in *Sp3*$^{+/-}$ embryos (Figure 4a).

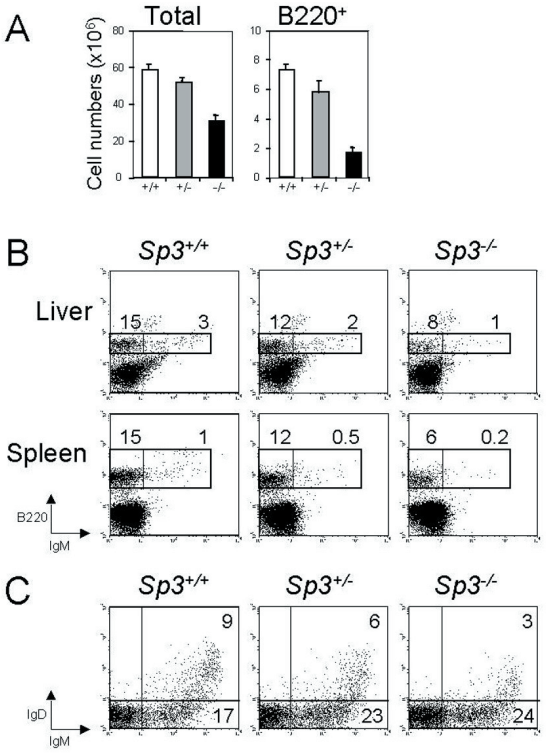


Figure 3. Impaired B-cell development in embryos lacking Sp3
(a) Numbers of total cells and B220^{+} cells in E18.5 liver of the indicated embryos, as determined by flow cytometry. (b) Flow cytometric analyses of the liver and spleen of the individual embryos. Single-cell supsensions were stained with B220 and IgM. Results are displayed as dot plots of lymphocyte gate cells; percentages of cells within the indicated gates are given. (c) Developmental progression of B-lineage cells in vitro. Total BM cells were cultured in the presence of IL-7 for 5 days, and recultured on S17 stromal cells in the absence of IL-7 to induce the formation of mature IgM^{+}IgD^{+} B cells. Cultured cells were stained for B220, IgM, and IgD. Results are displayed as B220 versus IgD dot plots. Data shown are from 4 to 15 embryos examined in each group.

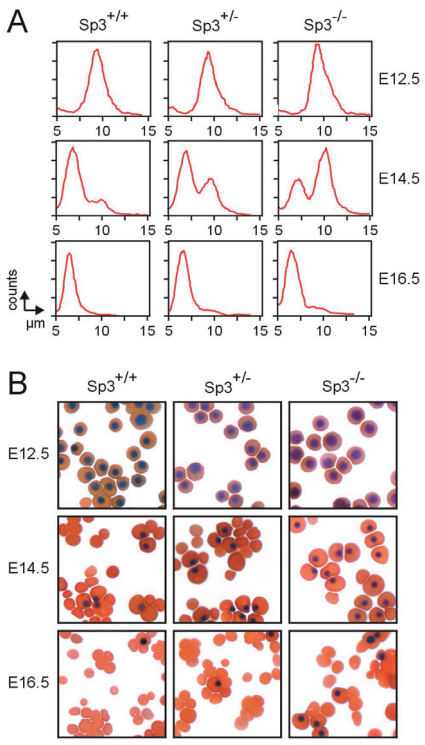


Figure 4. Delayed definitive erythropoiesis in *Sp3*-deficient embryos

(a) Analysis of the size distribution of circulating erythrocytes at different days of gestation. Yolk sac-derived erythrocytes (primitive) remain nucleated and have a diameter of approximately 10 µm. Fetal liver-derived erythrocytes (definitive) are enucleated and have a diameter of approximately 7 µm. Appearance of definitive erythroytes in the circulation is delayed in *Sp3*$^{-/-}$ embryos. (b) Cytospins were prepared from blood isolated from the indicated embryos and were stained with a combination of neutral benzidine and histologic dyes.[33] Nucleated primitive erythroytes are clearly distinguished from enucleated definitive erythrocytes; both cell types have a normal morphologic appearance in *Sp3*$^{-/-}$ embryos.

Sp3-deficient erythroid cells display a cell-autonomous differentiation defect

The observed delay in the appearance of circulating definitive erythrocytes could originate either from a delay in the colonization of the fetal liver by hematopoietic stem cells (HSCs) from the intraembryonic aorta-mesonephros-gonad (AGM) region[35] and the yolk sac or from an intrinsic defect in the developmental progression of definitive erythrocytes. To distinguish between these 2 possibilities, the numbers of the erythroid progenitors erythroid BFU-Es and CFU-Es were determined in the liver of *Sp3*$^{+/+}$, *Sp3*$^{+/-}$, and *Sp3*$^{-/-}$ embryos at E12.5 and E14.5. The numbers of BFU-Es and CFU-Es per fetal liver were reduced approximately 3-fold in *Sp3*$^{-/-}$ fetal livers (Figure 5a). However, when the values were corrected for the cellularity of the fetal livers, the ratios of BFU-Es and CFU-Es were comparable in the 3 groups of embryos (Figure 5b). These findings indicate that, despite the reduced cellularity, the architecture of the hematopoietic compartment of the *Sp3*$^{-/-}$ fetal livers is normal, excluding a critical role for Sp3 in the colonization of the liver with HSCs. We therefore conclude that the observed delay in the formation of definitive erythrocytes most likely results from an intrinsic developmental defect of definitive erythroid cells.

To study the effect of Sp3 on the developmental progression of definitive erythroid cells in detail, E14.5 fetal liver cells were grown for 3 days in a suspension culture system. Since erythroid precursors are taken out of their embryonic microenvironment and cultured under specific conditions for further differentiation, cell-autonomous defects in erythroid differentiation can be identified in this assay.[32] After 2 days of culture, 45% of the cells in the *Sp3*$^{+/+}$ cultures

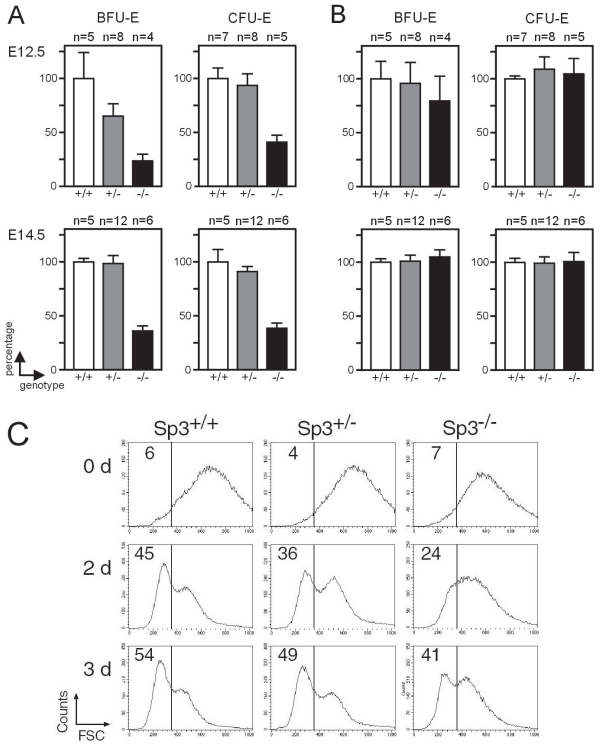


Figure 5. *Sp3*-deficient erythroid cells display a cell-autonomous differentiation defect
(a) The number of BFU-Es and CFU-Es per E12.5 and E14.5 fetal liver was determined for embryos with the indicated genotypes and gestational ages. The values in wild-type embryos were set at 100%. (b) Abundance of BFU-Es and CFU-Es in E12.5 and E14.5 *Sp3*-deficient embryos corrected for the cellularity of fetal livers. Wild-type values were set at 100%. (c) Fluorescence-activated cell sorter (FACS) analysis of fetal liver cells that were allowed to differentiate in vitro during 0, 2, and 3 days, using the suspension culture system. As the cells progress through maturation, they become smaller. Enucleated cells with small FSC values (lower than 360, as indicated) appear during culture at days 2 and 3.

were mature definitive erythrocytes, as compared with only 36% and 24% in $Sp3^{+/-}$ and $Sp3^{-/-}$ cultures, respectively (Figure 5c). On the third day of culturing, the differences between the 3 groups became less pronounced, but were still significant (Figure 5c).

In summary, these experiments show that Sp3-deficient erythroid cells display a cell-autonomous differentiation defect in vitro. This might explain the delay in the formation of circulating definitive erythrocytes observed in vivo in $Sp3^{-/-}$ embryos and, to a lesser extent, in $Sp3^{+/-}$ embryos.

Sp3-deficient fetal liver cells show severely reduced in vivo hematopoietic repopulation potential

The perinatal death of the $Sp3^{-/-}$ embryos precluded the analysis of adult hematopoiesis in the absence of Sp3. As we observed mild hematopoietic defects in Sp3-heterozygous embryos, we analyzed $Sp3^{+/-}$ mice at 8 weeks of age. However, when we compared the lymphoid, myeloid, and erythroid compartments of $Sp3^{+/-}$ and $Sp3^{+/+}$ mice by flow cytometry, we did not observe any significant differences (data not shown).

To further define the role of Sp3 in the differentiation of the various hematopoietic cell lineages, we investigated the ability of $Sp3^{-/-}$ fetal liver hematopoietic stem cells and progenitors to contribute to the lymphoid, myeloid, and erythroid lineages in an in vivo transplantation assay.[35] We transplanted E12.5 liver cells from $Sp3^{+/+}$, $Sp3^{+/-}$, and $Sp3^{-/-}$ embryos into irradiated

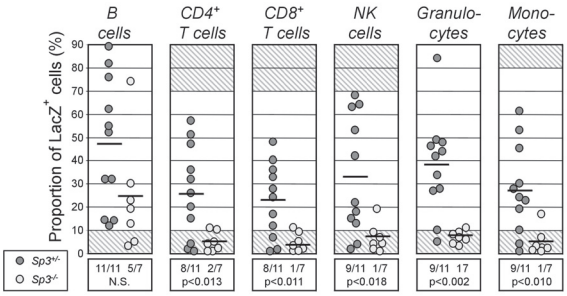


Figure 6. E12.5 *Sp3*-deficient fetal liver cells show reduced in vivo hematopoietic reconstitution
Analysis of the peripheral blood for *lacZ*-expressing cells at 6 weeks after transplantation in the indicated cell lineages in peripheral blood from mice that received *Sp3*$^{+/-}$ () or *Sp3*$^{-/-}$ () fetal liver cells. The mean number of injected cells per recipient was 2.6 x 10^5 (0.41 ± 0.17 embryo equivalents) for *Sp3*$^{+/-}$ fetal liver cells and 2.1 x 10^5 (0.58 ± 0.13 embryo equivalents) for *Sp3*$^{-/-}$ fetal liver cells. Fetal liver cells from 10 of 11 *Sp3*$^{+/-}$ embryos and 4 of 7 *Sp3*$^{+/-}$ embryos were transplanted into 2 mice, and in these cases the symbols show the average values in the 2 mice. In the other cases, the symbols represent the values in individual mice. Only recipients with more than 10% *lacZ*$^+$ cells are considered positive. For comparison, the proportions of *lacZ* expression in 3-month-old *Sp3*$^{+/-}$ heterozygous mice are indicated (approximately 70% in T and NK cells, approximately 80% in monocytes, and approximately 90% in B cells and granulocytes, values that would therefore reflect 100% engraftment).

adult female recipients. To correct for the differences in liver cellularity between the 3 genotypes, equal amounts of total liver cells were injected (approximately 2 x 10^5 cells). A limiting dose of syngeneic splenic cells was coinjected to aid the recipients in short-term survival following irradiation.

At 4 weeks after transplantation, recipient peripheral blood DNA was tested by PCR for the presence of the donor genetic marker, either the *Sp3* mutant allele for the *Sp3*$^{+/-}$ and *Sp3*$^{-/-}$ embryos, or the Y chromosome-specific marker YMT2 for the *Sp3*$^{+/+}$ embryos. The donor-derived genetic marker was detected to high levels (greater than 10%),[35] in all mice that received *Sp3*$^{+/+}$ cells (n = 4) or *Sp3*$^{+/-}$ cells (n = 11). The Sp3 mutant marker was also detected in 6 of 7 recipients receiving *Sp3*$^{-/-}$ fetal liver cells.

At 6 weeks after transplantation, we examined the extent of multilineage repopulation in mice receiving transplants of *Sp3*$^{+/-}$ and *Sp3*$^{-/-}$ fetal liver cells (n = 11 and n = 7, respectively) by flow cytometric analysis of peripheral blood leukocytes. The contribution of donor cells to individual hematopoietic cell lineages was quantified by calculating the percentage of *lacZ*-expressing cells within the following subpopulations: B220$^+$IgM$^{+/low}$ IgD$^{+/low}$ B cells, CD4$^+$ or CD8$^+$ T cells, NK1.1$^+$ NK cells, Mac-1$^+$Gr-1$^+$ granulocytes, and Mac-1$^+$Gr-1$^-$ monocytes (Figure 6). Although the percentages of engraftment varied between individual mice and between cell lineages, *Sp3*$^{+/-}$ cells were able to repopulate both lymphoid (B, T, and NK cells) and myeloid lineages (granulocytes and monocytes) in 8 of 11 recipients. In contrast, repopulation by *Sp3*$^{-/-}$ cells appeared to be restricted to the B-cell lineage (5 of 7 recipients). In those mice that showed repopulation of the B-cell lineage with *Sp3*$^{-/-}$ cells, the proportion of *lacZ*$^+$ cells was on average lower, when compared with the *Sp3*$^{+/-}$ group of mice. Only one mouse showed engraftment of *Sp3*$^{-/-}$ cells in the populations of CD4$^+$ and CD8$^+$ T cells, NK cells, granulocytes, and monocytes,

but again the percentages of *lacZ*$^{+}$ cells within these cell populations were low and did not exceed 20%.

In summary, from these analyses, we conclude that *Sp3*$^{-/-}$ fetal liver cells have a reproducible deficiency in short-term repopulation capacity of the hematopoietic system. In this respect, the presence of Sp3 appears to be less critical for reconstitution of the B-cell lineage.

_Sp3_-deficient fetal liver cells display a specific defect in the ability to repopulate the erythroid and myeloid lineages

The observed reduced repopulation capacity of *Sp3*$^{-/-}$ E12.5 fetal liver cells could either reflect a delayed development of mature lymphoid and myeloid cells from HSCs or multipotent progenitors or, alternatively, result from specific developmental arrests within these cell lineages.

To distinguish between these 2 possibilities, we analyzed, in more detail, the extent of hematopoietic repopulation in mice that received *Sp3*$^{+/-}$ (n = 3) and *Sp3*$^{-/-}$ (n = 3) fetal liver cells at 3 months after transplantation. The percentages of *lacZ*$^{+}$ cells were quantified within specific cell lineages present in hematopoietic tissues, including thymus, spleen, blood, bone marrow, and peritoneal cavity. At 3 months after transplantation, repopulation by *Sp3*$^{+/-}$ cells appeared to be almost complete (Figure 7), as in all analyzed mice the percentages of *lacZ*$^{+}$ cells within the lymphoid, myeloid, and erythroid cell lineages were close to the values found in control adult *Sp3*$^{+/-}$ mice. In contrast to our results at 6 weeks after transplantation, at 3 months after transplantation repopulation by *Sp3*$^{-/-}$ fetal liver cells was no longer confined to B cells, as the T-cell lineage also displayed significant repopulation by *Sp3*$^{-/-}$ donor cells: the fractions of *lacZ*$^{+}$ cells were 30% ± 18% and 15% ± 5% for CD4^{+} and CD8^{+} peripheral blood T cells, respectively (not shown). Figure 7 shows the analyses of the animal receiving transplants of *Sp3*$^{-/-}$ cells in which the highest percentages of *lacZ*$^{+}$ cells were observed. The thymocytes of the recipients grafted with *Sp3*$^{+/-}$ or *Sp3*$^{-/-}$ cells showed a similar pattern of modulated lacZ expression as was previously found in E18.5 thymus (Figures 1a,7a). Also in the spleen, *Sp3*$^{-/-}$ donor-derived cells contributed to the mature CD4^{+} and CD8^{+} T-cell populations. Moreover, *Sp3*$^{-/-}$ CD4^{+} T cells were not different from *Sp3*$^{+/-}$ CD4^{+} T cells in their ability to differentiate in vivo into antigen-experienced CD45RB^{low} T cells[39] (Figure 7b). *Sp3*$^{-/-}$ cells were less capable of repopulation in the NK-cell lineage (the proportions of *lacZ*$^{+}$ cells were 10% ± 5% (n = 3) versus 56% ± 19% in the group of mice with transplants of *Sp3*$^{+/-}$ cells). *Sp3*$^{-/-}$ cells contributed substantially to all peripheral B-cell subpopulations, including mature IgM^{low}IgD^{high} follicular B cells and CD21^{high} CD23^{-} marginal zone cells in the spleen, and CD19^{high}CD5^{+} B-1 peritoneal B cells (Figure 7b and data not shown), as well as to the B-cell compartment in the bone marrow (Figure 7c).

Even though the donor-derived *Sp3*$^{-/-}$ cells showed a substantial contribution to the lymphoid lineages, in the same recipient mice these cells did not repopulate the erythroid or myeloid lineages. Very low proportions of *lacZ*$^{+}$ cells were found in the large FSC Ter119^{+} erythroid precursors or the Ly6-C^{med} granulocyte and Ly6-C^{high} monocyte precursors in the bone marrow (Figure 7c). Also mature granulocytes in the peripheral blood and mature macrophages in the

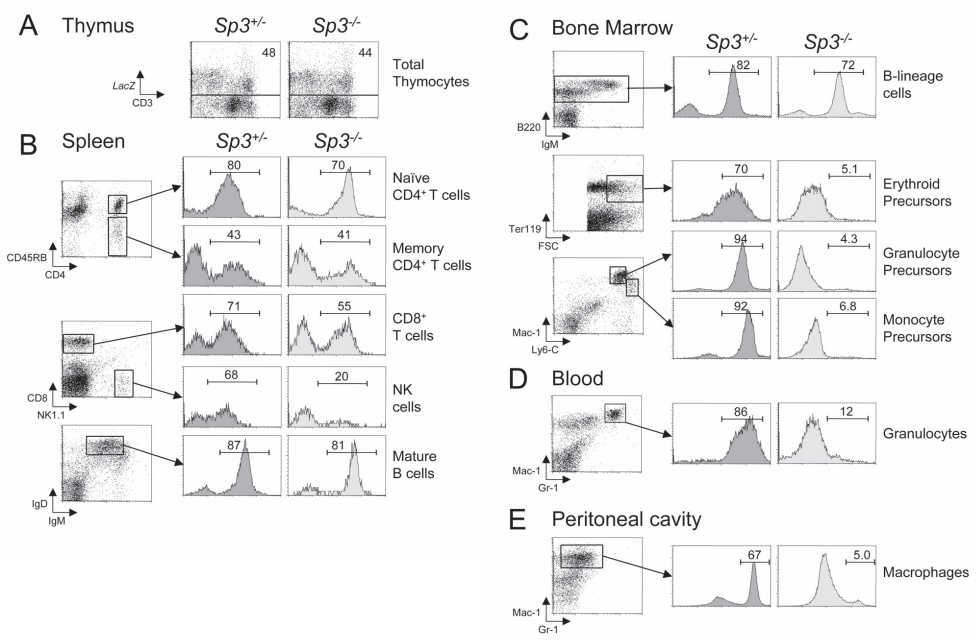


Figure 7. Absence of erythroid and myeloid repopulation by *Sp3*$^{-/-}$ fetal liver cells in an in vivo transplantation assay

(a) Analysis for CD3 expression and *lacZ* activity in total thymocytes from *Sp3*$^{+/-}$ and *Sp3*$^{-/-}$ transplant recipients. (b-e) Total cell suspensions from spleen (panel b), bone marrow (panel c), peripheral blood (panel d), and peritoneal cavity (panel e) were analyzed for lymphoid-, erythroid-, or myeloid-specific marker expression (left). In *Sp3*$^{+/-}$ and *Sp3*$^{-/-}$ transplant recipients, the indicated subpopulations were gated and analyzed for donor-derived *lacZ* expression (right). The results for transplanted *Sp3*$^{-/-}$ cells are from the animal in which the highest percentages of *lacZ*$^{+}$ cells were observed. *LacZ* expression data are displayed as histograms; the numbers are the proportions of *lacZ*$^{+}$ cells in which background values in wild-type mice were lower than 4%.

peritoneum did not contain significant populations of *Sp3*$^{-/-}$ *lacZ*$^{+}$ cells (Figure 7d-e).

From these findings, we conclude that at 3 months after transplantation, E12.5 *Sp3*$^{-/-}$ fetal liver cells can substantially repopulate the B- and T-cell lineages in lethally irradiated recipients. *Sp3*$^{-/-}$ cells were able to repopulate the NK-cell compartment, although the efficiency was very low. In strong contrast, the absence of Sp3 precluded significant repopulation of the erythroid- and myeloid-cell lineages in the in vivo transplantation assay.

Conclusions

In this report, we have studied the impact of the absence of the widely expressed transcription factor Sp3 on the developing hematopoietic system in the mouse. We demonstrate that lymphopoiesis, erythropoiesis, and myelopoiesis are all affected in *Sp3* knockout embryos. Since these embryos are retarded in development,[15] our observations might merely reflect the general delay in developmental progression. However, several lines of evidence argue against

this hypothesis: (1) The finding of phenotypic alterations in erythroid cells in our in vitro cell culture experiments point toward an intrinsic defect in erythropoiesis in Sp3-*null* cells. (2) Even if the general developmental retardation is taken into account, the appearance of definitive erythrocytes in the circulation of Sp3-*null* embryos is delayed, and the B- and T-cell precursor populations are reduced in size. (3) The in vivo transplantation assay revealed differential effects of the absence of Sp3 for the individual cell lineages: delayed but almost complete repopulation of the B- and T-lymphoid compartment, and no significant repopulation in the erythroid and myeloid lineages. (4) The observed intermediate phenotype of *Sp3*$^{+/-}$ embryos with regard to the appearance of definitive erythrocytes suggests a dosage effect. Effects of haploinsufficiency on hematopoiesis have been reported before, for instance, for EKLF[40] and acute myeloid leukemia 1 (AML1),[41] and are a strong indication for a direct role of the factor in the lineage affected. Therefore, our data collectively suggest that Sp3 deficiency has a specific impact on hematopoiesis.

Sp/XKLF family members and erythropoiesis

The hematopoietic defects of Sp3-*null* mice are subtle, and it appears unlikely that hampered hematopoiesis contributes significantly to the perinatal lethality. Severe anemia resulting from insufficient production of functional erythroid cells is the cause of embryonic lethality in the knockout of another Sp/XKLF family member, EKLF. In these mice, definitive erythropoiesis is defective owing to failure to activate expression of the adult β-globin genes.[8,9] Surprisingly, the embryonic globins and the adult α-globins are expressed normally in the EKLF knockouts. Many, if not all, erythroid-specific genes contain functionally important Sp/XKLF–binding sites in their promoters and other regulatory elements. Yet the transcription factors by which these genes are activated in vivo are still largely unknown, owing to the fact that the Sp/XKLF family comprises more than 20 different transcription factors. At least 5 of these (Sp1, Sp3, BKLF, EKLF, and fetal KLF [FKLF])[1] are expressed in erythroid cells. BKLF knockout mice do not appear to display an overt erythroid phenotype, but BKLF is known to be up-regulated in EKLF-*null* cells.[8,11] This suggests that there might be overlapping functionality between BKLF and EKLF. Analysis of compound mutant mice would be required to clarify this issue. We have generated *Sp1* knockout mice in our laboratory, but the early embryonic lethality precludes the analysis of definitive hematopoiesis in Sp1-deficient embryos.[14] We have now created a conditional knockout allele of the *Sp1* mutation. Preliminary data on erythroid-specific knockouts indicate that Sp1 is essential for primitive and definitive erythropoiesis (U. Jägle and S.P., unpublished results, December 2001). Our analysis of *Sp4* knockout mice has failed to demonstrate any hematopoietic defect, apart from the reduced cellularity of the thymus and spleen.[18] This is thought to reflect a defect in the hormonal pituitary-gonadal axis, since it is known that such defects correlate with a reduced size of the thymus and delayed sexual maturation, as observed in *Sp4* knockout mice.[18]

Sp3 and embryonic/fetal erythropoiesis

The analysis of erythropoiesis in Sp3-*null* mice presented here demonstrates a role for Sp3 in the transition from primitive to definitive erythropoiesis, and in the progression of erythroid maturation. Moreover, the lack of detectable repopulation of the erythroid compartment in the bone marrow by Sp3-deficient fetal liver cells in the transplantation experiments in vivo, together with the observed cell-autonomous differentiation defect in vitro, indicates that Sp3 function is essential for normal erythropoiesis. However, we did not find evidence for a critical role of Sp3 in the developmental timing of fetal- to adult-globin switching in vivo. In crosses of Sp3-deficient mice with transgenic mice containing the human globin cluster,[42] the switch from γ- to β-globin expression takes place between E11.5 and E13.5, irrespective of the Sp3 genotype (P.B. and S.P., unpublished results, September 2001).

Sp3 and lymphopoiesis

In the T-cell lineage, it is of interest to note that *lacZ* staining indicates down-regulation of Sp3 expression upon transition of thymocytes from the DP to the SP cell stage. Of course, this may imply that Sp3 expression directly regulates genes that promote apoptosis of DP cells. However, Sp3 differs biochemically from the other Sp factors in that it contains an inhibitory domain.[43] Sp3 may therefore also function as a repressor of Sp-mediated transcription on some promoters.[1,43] Thus, down-regulation of Sp3 at the DP stage may shift the balance of factors bound to GT boxes toward other Sp/XKLF family members. In this context, Sp3 could modulate the activity of the LKLF, which is induced at the progression from DP into SP and appears to play a critical role in programming the quiescent phenotype of SP thymocytes.[13] However, Sp3 does not appear to be an essential regulator at this stage of T-cell development, as the observed partial arrest of T development at the DP stage in E18.5 $Sp3^{-/-}$ embryos was not paralleled by a defect at this stage in the mice in the transplantation group that received $Sp3^{-/-}$ fetal liver cells. The $lacZ^{+}$ DP and DD subpopulations in the mice receiving transplants of $Sp3^{+/-}$ and $Sp3^{-/-}$ cells did not differ in FSC characteristics or the level of CD3 expression (data not shown). Also, the finding of similar contributions of $Sp3^{+/-}$ and $Sp3^{-/-}$ cells to the $CD45RB^{low}$ population of antigen-experienced $CD4^{+}$ T cells in the spleen indicates that $Sp3^{-/-}$ T cells can function normally and argues against a crucial role of Sp3 in positive selection in the thymus. Rather, the reduced size of the T-cell compartment in E18.5 embryos and the decreased capacity and kinetics to repopulate the T-cell lineage in the in vivo transplantation assay suggest a subtle defect in the expansion or generation of early T-cell progenitors. As the defects in the B-cell lineage are similar, it is attractive to speculate that Sp3 deficiency affects a common lymphoid progenitor. However, it is still unknown whether the common lymphoid progenitor that has been identified in adult mouse bone marrow also exists in fetal liver.[44,45]

Sp3 and adults eythropoiesis/myelopoiesis

The transplantation experiments demonstrate that $Sp3^{-/-}$ fetal liver cells have a severely impaired capacity to repopulate the erythroid and myeloid compartments. Our analysis reveals

the absence of *Sp3*$^{-/-}$ myeloid cells at all peripheral sites investigated. In the bone marrow, we found that *Sp3*$^{-/-}$ cells fail to contribute to the erythroid/myeloid progenitor pool. Since these cells are contributing quite efficiently to the lymphoid system in the same animals, we conclude that Sp3 plays an important role in the development of erythroid/myeloid progenitors. Possibly, Sp3 acts at the early level of the common progenitors to the erythroid and myeloid lineages. Clearly, more experiments are needed to further define the function of Sp3 in myeloid development. In this regard, it is of interest to note that mice deficient for the Sp/XKLF family member BKLF develop a myeloproliferative disorder,[46] raising the possibility of antagonistic functions of Sp3 and BKLF in myelopoiesis.

Conclusions

Here, we describe the effect of the Sp3-*null* mutation on hematopoiesis. Our findings, in particular the in vivo transplantation assay, showed that the absence of Sp3 results in cell-autonomous differentiation defects in the erythroid and myeloid cell lineage. Sp/XKLF factors share 3 highly conserved DNA-binding zinc fingers, and most hematopoietic cell populations express multiple members of the Sp/XKLF family. In conjunction with the phenotypes of the Sp/XKLF knockouts for which hematopoietic defects have been reported in this paper and elsewhere,[8,9,11,13,47] it can be anticipated that Sp/XKLF factors have overlapping functions in hematopoietic cells. We are currently generating compound mutants to address this issue.

Materials and Methods

Mice

Embryos were derived from timed matings of *Sp3*$^{+/-}$ x *Sp3*$^{+/-}$ mice.[15] Genotyping was performed by polymerase chain reaction (PCR), with the use of the following 3 primers: a sense primer in the *Sp3* gene amplifying the wild-type allele (5'-GCGTGCAAGCCAGTGGTC-3'); a sense primer in the Neo gene amplifying the knockout allele (5'-AGCGCATCGCCTTCTATCG-3'); and a common antisense primer in the *Sp3* gene (5'-GGACGATTCTATGCCTCC-3'). PCR conditions were 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles.

Flow cytometric analysis

The preparation of single-cell suspensions, determination of β-galactosidase activity by means of fluorescein-di-β-D-galactopyranoside (FDG), antibody incubations, and 3- or 4-color cytometry have been described previously.[27] The following monoclonal antibodies were obtained from Pharmingen (San Diego, CA): fluorescein isothiocyanate (FITC)-conjugated anti-B220/RA3-6B2 and anti-CD3; phycoerythrin (PE)-conjugated anti-CD4, anti-CD5, anti-CD11b/macrophage 1 (Mac-1), anti-CD19, anti-C23, anti-CD25/IL-2R (clone 3C), anti-CD43/Ser7, anti-CD45RB, anti-CD69, anti–natural killer cell 1.1 (anti-NK1.1), and anti-Ter119; biotinylated anti-IgM, anti-CD4, and anti-CD8; CyChrome–conjugated anti-CD8, anti-CD44, and anti-B220/RA-6B2; peridinin chlorophyll protein (Per-CP)-conjugated anti-CD11b/Mac1; and allophycocyanin (APC)-conjugated anti-CD3, anti-CD5, and anti-CD4. Southern Biotechnology Associates (Birmingham, AL) supplied PE-conjugated anti-IgD.

Anti-CD8/53-6.7, anti-CD21, anti-Gr-1/RB6-8C5, and ER-MP20/Ly6-C[28] were purified monoclonal antibodies conjugated to biotin according to standard procedures. Secondary antibodies used were Tricolor- or PE-conjugated streptavidin (Caltag Laboratories, Burlingame, CA) or CyChrome- or APC-conjugated streptavidin (Pharmingen).

In vitro culture of fetal liver-derived B and erythroid cells

Primary pre-B-cell cultures were essentially performed as described previously.[29,30] In brief, fetal liver cells were cultured in Iscove modified Dulbecco medium (IMDM) medium, supplemented with 10% heat-inactivated fetal calf serum (FCS) at 2 to 3 x 10^6 cells per well in 24-well plates in the presence of 100 U/mL recombinant IL-7 (R&D Systems, Minneapolis, MN). After 5 days of culture, cells were harvested and recultured on S17 stromal cells with or without 100 U/mL IL-7 for 48 hours.

For suspension culture assays, fetal livers were collected from embryos and disaggregated to single-cell suspensions; then, 5 x 10^4 cells were placed in 20-µL drops suspended on the lid of a Petri dish (Lindeboom et al, manuscript in preparation).

Colony assays

Colony assays were performed essentially as described.[31,32] Fetal livers were disaggregated into single cells by pippeting and were plated out in methyl cellulose containing 1 U/mL erythropoietin (Epo) at a cell density of 15 x 10^5 and 3 x 10^5/mL for erythroid colony-forming units (CFU-Es) and erythroid burst-forming units (BFU-Es), respectively. The appearance of colonies was scored after 3 (CFU-Es) and 9 (BFU-Es) days.

Analysis of peripheral blood

E12.5, E14.5, and E16.5 embryos were dissected and bled on a dish to collect the fetal blood in 1 x phosphate-buffered saline (PBS) containing 0.5 mM EDTA (ethylenediaminetetraace tic acid). Cell size distributions were determined on a Casy1 instrument (Schärfe System, Reutlingen, Germany). Aliquots of the blood were loaded on a cytofunnel and spun at 1000 rpm for 2 minutes on microscope slides. The preparations were left to air dry and stained with a combined histologic and neutral benzidine-staining procedure.[33]

In vivo transplantation assay

Fetal liver cell suspensions, prepared by treatment with 0.125% wt/vol collagenase for 1 hour at 37°C in PBS with 10% FCS, were transferred intravenously into 8- to 10-week-old C57BL/6 female mice, which were exposed to a split dose of 9 Gy at a 3-hour interval by a ^{137}Cs source. To provide short-term survival, 2 x 10^5 C57BL/6 female splenocytes were coinjected. Mice were maintained in filter-top cages and received 0.16% neomycin-supplemented water.[34,35] Donor tissues were examined for *Sp3* genotype and sex (Y chromosome) by DNA PCR of somite tissue or body remnants from dissected embryos. Donor-derived engraftment was examined by semiquantitative PCR of DNA from peripheral blood with the use of *Sp3* wild-type and mutant-specific primers, or primers for the male-specific *YMT2* gene.[34,35]

Acknowledgements and footnotes

We thank H. Dronk from the Erasmus MC animal facility and G. Dingjan from Erasmus MC Department of Immunology, Rotterdam, The Netherlands, for their assistance.
Submitted June 21, 2002; accepted February 25, 2003.
Prepublished online as *Blood* First Edition Paper, April 3, 2003; DOI 10.1182/blood-2002-06-1848.
Supported by the Netherlands Organization for Scientific Research (NWO) (R.W.H., F.G., and S.P.) and the Deutsche Forschungsgemeinschaft (DFG) (G.S.).
The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

References

1. Philipsen, S. & Suske, G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* **27**, 2991-3000 (1999).
2. Costa, M. W. & Atchison, M. L. Identification of an Spl-like element within the immunoglobulin kappa 3' enhancer necessary for maximal enhancer activity. *Biochemistry* **35**, 8662-9 (1996).
3. Rotheneder, H., Geymayer, S. & Haidweger, E. Transcription factors of the Sp1 family: interaction with E2F and regulation of the murine thymidine kinase promoter. *J Mol Biol* **293**, 1005-15 (1999).
4. Hirano, F. et al. Functional interference of Sp1 and NF-kappaB through the same DNA binding site. *Mol Cell Biol* **18**, 1266-74 (1998).
5. Merika, M. & Orkin, S. H. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. *Mol Cell Biol* **15**, 2437-47 (1995).
6. Birnbaum, M. J. et al. Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. *Biochemistry* **34**, 16503-8 (1995).
7. Dennig, J., Hagen, G., Beato, M. & Suske, G. Members of the Sp transcription factor family control transcription from the uteroglobin promoter. *J Biol Chem* **270**, 12737-44 (1995).
8. Perkins, A. C., Sharpe, A. H. & Orkin, S. H. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature* **375**, 318-22 (1995).
9. Nuez, B., Michalovich, D., Bygrave, A., Ploemacher, R. & Grosveld, F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature* **375**, 316-8 (1995).
10. Gillemans, N. et al. Altered DNA-binding specificity mutants of EKLF and Sp1 show that EKLF is an activator of the beta-globin locus control region in vivo. *Genes Dev* **12**, 2863-73 (1998).
11. Perkins, A. C., Yang, H., Crossley, P. M., Fujiwara, Y. & Orkin, S. H. Deficiency of the CACC-element binding protein, BKLF, leads to a progressive myeloproliferative diease and impaired expression of SHP-1. *Blood, Supplement 1* **90**, 575a (1997).
12. Kuo, C. T. et al. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes Dev* **11**, 2996-3006 (1997).
13. Kuo, C. T., Veselits, M. L. & Leiden, J. M. LKLF: A transcriptional regulator of single-positive T cell quiescence and survival. *Science* **277**, 1986-90 (1997).
14. Marin, M., Karis, A., Visser, P., Grosveld, F. & Philipsen, S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* **89**, 619-28 (1997).
15. Bouwman, P. et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. *Embo J* **19**, 655-61 (2000).
16. Gollner, H., Dani, C., Phillips, B., Philipsen, S. & Suske, G. Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**, 77-83 (2001).
17. Nguyen-Tran, V. T. et al. A novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages. *Cell* **102**, 671-82 (2000).
18. Gollner, H. et al. Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. *Genes Cells* **6**, 689-97 (2001).
19. Kingsley, C. & Winoto, A. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. *Mol Cell Biol* **12**, 4251-61 (1992).
20. Sikes, M. L., Gomez, R. J., Song, J. & Oltz, E. M. A developmental stage-specific promoter directs germline transcription of D beta J beta gene segments in precursor T lymphocytes. *J Immunol* **161**, 1399-405 (1998).
21. Lin, J. X. & Leonard, W. J. The immediate-early gene product Egr-1 regulates the human interleukin-2 receptor beta-chain promoter through noncanonical Egr and Sp1 binding sites. *Mol Cell Biol* **17**, 3714-22

(1997).
22. Malone, C. S., Omori, S. A. & Wall, R. Silencer elements controlling the B29 (Igbeta) promoter are neither promoter- nor cell-type-specific. *Proc Natl Acad Sci U S A* **94**, 12314-9 (1997).
23. Malone, C. S., Patrone, L. & Wall, R. An essential octamer motif in the mb-1 (Igalpha) promoter. *Mol Immunol* **37**, 321-8 (2000).
24. Himmelmann, A., Thevenin, C., Harrison, K. & Kehrl, J. H. Analysis of the Bruton's tyrosine kinase gene promoter reveals critical PU.1 and SP1 sites. *Blood* **87**, 1036-44 (1996).
25. Rohrer, J. & Conley, M. E. Transcriptional regulatory elements within the first intron of Bruton's tyrosine kinase. *Blood* **91**, 214-21 (1998).
26. Friedman, A. D. Transcriptional regulation of granulocyte and monocyte development. *Oncogene* **21**, 3377-90 (2002).
27. Hendriks, R. W. et al. Inactivation of Btk by insertion of lacZ reveals defects in B cell development only past the pre-B cell stage. *Embo J* **15**, 4862-72 (1996).
28. de Bruijn, M. F. et al. Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. *Eur J Immunol* **24**, 2279-84 (1994).
29. Rolink, A., Streb, M. & Melchers, F. The kappa/lambda ratio in surface immunoglobulin molecules on B lymphocytes differentiating from DHJH-rearranged murine pre-B cell clones in vitro. *Eur J Immunol* **21**, 2895-8 (1991).
30. Dingjan, G. M. et al. Bruton's tyrosine kinase regulates the activation of gene rearrangements at the lambda light chain locus in precursor B cells in the mouse. *J Exp Med* **193**, 1169-78 (2001).
31. Wong, P. M., Chung, S. W., Chui, D. H. & Eaves, C. J. Properties of the earliest clonogenic hemopoietic precursors to appear in the developing murine yolk sac. *Proc Natl Acad Sci U S A* **83**, 3851-4 (1986).
32. Whyatt, D. et al. An intrinsic but cell-nonautonomous defect in GATA-1-overexpressing mouse erythroid cells. *Nature* **406**, 519-24 (2000).
33. Beug, H., Palmieri, S., Freudenstein, C., Zentgraf, H. & Graf, T. Hormone-dependent terminal differentiation in vitro of chicken erythroleukemia cells transformed by ts mutants of avian erythroblastosis virus. *Cell* **28**, 907-19 (1982).
34. Muller, A. M. & Dzierzak, E. A. ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients. *Development* **118**, 1343-51 (1993).
35. Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. & Dzierzak, E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301 (1994).
36. Singer, A. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. *Curr Opin Immunol* **14**, 207-15 (2002).
37. Lucas, B. & Germain, R. N. Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4+CD8+ thymocyte differentiation. *Immunity* **5**, 461-77 (1996).
38. Russell, E. S. & Bernstein, S. E. Blood and blood formation. *Biology of the laboratory mouse, E.L. Green, New York*, 351-372 (1966).
39. Dutton, R. W., Bradley, L. M. & Swain, S. L. T cell memory. *Annu Rev Immunol* **16**, 201-23 (1998).
40. Wijgerde, M. et al. The role of EKLF in human beta-globin gene competition. *Genes Dev* **10**, 2894-902 (1996).
41. Cai, Z. et al. Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity* **13**, 423-31 (2000).
42. Strouboulis, J., Dillon, N. & Grosveld, F. Developmental regulation of a complete 70-kb human beta-globin locus in transgenic mice. *Genes Dev* **6**, 1857-64 (1992).
43. Hagen, G., Muller, S., Beato, M. & Suske, G. Sp1-mediated transcriptional activation is repressed by Sp3. *Embo J* **13**, 3843-51 (1994).
44. Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-72 (1997).
45. Katsura, Y. Redefinition of lymphoid progenitors. *Nat Rev Immunol* **2**, 127-32 (2002).
46. Turner, J. & Crossley, M. Basic Kruppel-like factor functions within a network of interacting haematopoietic transcription factors. *Int J Biochem Cell Biol* **31**, 1169-74 (1999).
47. Wani, M. A., Means, R. T., Jr. & Lingrel, J. B. Loss of LKLF function results in embryonic lethality in mice. *Transgenic Res* **7**, 229-38 (1998).

CHAPTER 3

Chapter 3

Transcription factor Sp3 has a crucial role in cardiac development

Pieter Fokko van Loo,[1] Bert Wisse,[2] Frank Grosveld,[1] Guntram Suske,[3] Sjaak Philipsen,[1] and Adriana Gittenberger-de Groot

[1] Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands
[2] Department of Anatomy and Embryology, LUMC, Leiden, The Netherlands
[3] Institut für Molekularbiologie und Tumorforschung Philipps-Universität Marburg, Germany

Abstract

Mice lacking the ubiquitously expressed zinc-finger transcription factor specificity protein 3 (Sp3) die prenatally or shortly after birth. To elucidate one possible cause of the mortality of Sp3-*null* embryos, we analysed the potential role of Sp3 in embryonic heart development. Our study revealed defective looping resulting in a wide inner curvature of *Sp3*$^{-/-}$ hearts at embryonic day 10.5 (E10.5), causing severe abnormalities in cardiac morphology in all Sp3-*null* embryos at E12.5. At E14.5, the majority of the Sp3-*null* mutant hearts have developed a double outlet right ventricle and a spectrum ranging from overriding tricuspid valve to double inlet left ventricle. The compact myocardium is thin with many perforations allowing direct sub-endocardial to sub-epicardial contact. To position the transcription factor Sp3 in the heart development pathway, we analysed the expression of genes known to be essential for normal murine heart development, including Nkx2-5, Gata4, Anf, and Carp. The expression patterns of the genes analysed were not altered in developing *Sp3*$^{-/-}$ hearts, with the exception of the gene encoding Carp. Carp becomes slightly down-rgulated in *Sp3*$^{-/-}$ hearts at E12.5, and is almost completely absent in E14.5 *Sp3*$^{-/-}$ hearts. Analysis of the *Carp* promoter reveals that Sp3 can bind to the *Carp* promoter region, suggesting that Carp is directly regulated by Sp3, most likely in cooperation with known cardiac-specific transcriptional activators such as Nkx2-5 and Gata4. Our results demonstrate a crucial role for transcription factor Sp3 in myocardial differentiation and outflow remodelling.

Introduction

Specificity protein 3 (Sp3) is a family member of the transcription factor family Sp/XKLF (specificity protein/x Krüppel-like factor) that is characterized by three conserved Krüppel-like Cys_2-His_2 zinc-fingers, located in the C-terminus of the Sp/XKLF proteins. The three fingers form the DNA-binding domain that recognizes the GC-(ggGGCGGgg) box and the related GT-(ggGGTGTgg) box, present in regulatory regions of many housekeeping- and tissue-specific genes. The Sp-subclass members Sp1, Sp3, and Sp4 bind with similar specificity and affinity to GC- and GT-boxes, and they all contain two glutamine-rich activation domains in their N-terminus. Sp1 and Sp3 are widely expressed, whereas Sp4 is most prominently expressed in neuronal tissues (reviewed in references [1,2]). *Sp1* knockout embryos are severely growth retarded and die after E10.5.[3] Although Sp3 is highly homologous to Sp1 in terms of its expression pattern and protein sequence, its biological function is quite different from that of Sp1. In a mixed genetic background of C57Bl/6 and 129/Ola, Sp3-*null* mutant mice die invariably immediately after birth.[4] Their immediate post-partum lethality likely results from respiration failure. However, only minor morphological abnormalities were observed in Sp3-*null* lungs, and expression of essential lung-specific genes, such as surfactant proteins, is unaltered.[4] *Sp3*$^{-/-}$ fetuses have a defect in tooth development, reflected by absence of ameloblast-specific transcripts, and their skeletal ossification is impaired.[5] Furthermore, absence of Sp3 results in cell-autonomous differentiation defects in the erythroid- and myeloid cell lineages, indicating that Sp3 also has a function in normal hematopoiesis.[6] *Sp4*-null mice have initially a reduced body weight and display an increased incidence of early postnatal mortality.[7,8] The latter appears to be related to disturbed development of the cardiac conduction system.[9]

Recently, we found a high mortality rate of *Sp3*$^{-/-}$ fetuses in the C57Bl/6 background. The majority of these null mutants die before E18.5, with an increased mortality rate already observed at E16.5. The increased prenatal mortality rate suggests that lung failure may not be the main cause of the previously described immediate postnatal lethality of *Sp3*$^{-/-}$ fetuses.

Congenital heart defects are a common cause of pre- and perinatal mortality.[10-12] The heart is the first organ formed during mammalian embryogenesis and develops from the anterior lateral mesoderm. The cells of the mesoderm become, via signalling from adjacent endoderm, committed to cardiac fate and start to express cardiac-specific genes (reviewed in reference [13]). Soon thereafter, the newly formed cardiac cells assemble along the ventral midline of the embryo into a beating linear heart tube, around E7.5 in the mouse. Next, the linear heart tube undergoes rightward looping, a process that is essential to position the in- and outflow tracts in close proximity to the developing four chambers. These heart chambers later acquire their own specific morphological characteristics. Further proliferation of cardiomyocytes allows the heart to increase blood load during embryonic growth. A complex transcriptional network controls these processes (reviewed in references [13,14]).

To investigate the potential cause of the high prenatal mortality rate of *Sp3*$^{-/-}$ embryos, we

performed a detailed morphological investigation of heart development. This was combined with a study of the expression patterns of early cardiac genes (including Nkx2-5, Gata4, and Tbx5) and late cardiac genes (including Irx4, Carp, Anf, Msx2, and Tgfβ2), as a first approach to determine the position of Sp3 in the cardiac regulatory pathway.

Results

Sp3$^{-/-}$ fetuses display severe cardiac malformations

As we observed a high mortality rate of *Sp3*$^{-/-}$ fetuses during pregnancy in the C57Bl/6 background, we decided to investigate the development of *Sp3*-deficient embryos in more detail. In addition to the earlier reported growth retardation of the *Sp3*$^{-/-}$ fetuses, we often observed edematic Sp3-*null* mutants around E14.5 (Figure 1a,d). This finding suggests that cardiac dysfunction may contribute to the increased mortality rate of *Sp3*$^{-/-}$ fetuses. To investigate this possibility, serial sections of E10.5 (n=14), E12.5 (n=12), and E14.5 (n=17) *Sp3*$^{-/-}$ embryos were examined. A wide inner curvature was found in E10.5 and E12.5 *Sp3*$^{-/-}$ hearts. At E12.5, the wide inner curvature of *Sp3*$^{-/-}$ hearts was reflected in delayed progression of in- and outflow tract remodeling. The pulmonary trunk and the aorta were standing side by side in all E12.5 *Sp3*$^{-/-}$ hearts examined (Figure 1b,e) and were connected to a greater extent to the right ventricle as compared to normal. In 8/12 E12.5 mutant hearts, both future atria were most prominently connected to the left ventricle. In wild-type littermates, the right atrio-ventricular (future tricuspid orifice) connection is to the right ventricle (Figure 1c,f).

In addition, we found a very primitive heart structure in three *Sp3*$^{-/-}$ E12.5 hearts. These

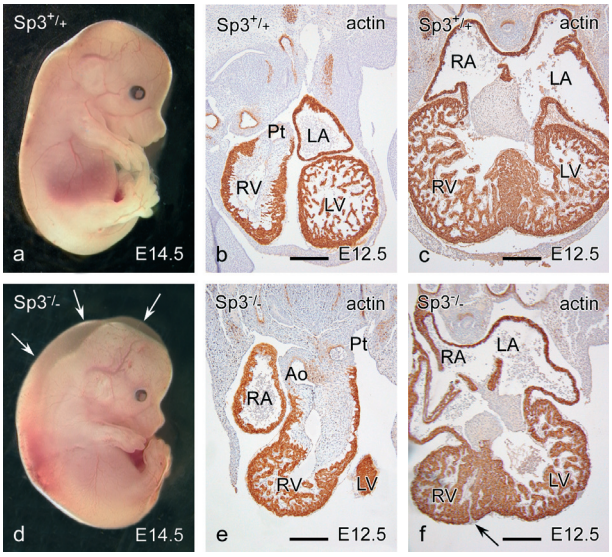


Figure 1. Cardiac and cardiac-related malformations in *Sp3*$^{-/-}$ fetuses
(a,d) The majority of *Sp3*$^{-/-}$ fetuses are edematic at E14.5, indicating that the Sp3 *null* mutant has a cardiac dysfunction (in d indicated by arrows). (b,c,e,f) E12.5 hearts stained for actin. Comparison of (b,c) with (e,f) shows a side by side pulmonary trunk and aorta as well as an aorta coming form the right ventricle (b,e) and a right atrium that is connected to a greater extent to the left ventricle in the *Sp3*$^{-/-}$ fetus compared to wild-type littermates (c,f). Myocardium of *Sp3*-deficient heart is perforated, allowing direct sub-endocardial to sub-epicardial contact, indicated by arrow in (f). Ao: aorta; Pt: pulmonary trunk; RV: right entricle; LV: left ventricle; RA: right atrium; LA: left atrium. Scale bars: 100 µm.

hearts had a single inlet and outlet, one common ventricle, and no expanding ventricular septum separating the future ventricles. In 4/12 of the E12.5 *Sp3*$^{-/-}$ hearts there was a common arterial trunk, a situation in which the outflow tract is not septated into the ascending aorta and the pulmonary trunk.

The wide inner curvature established at the earlier embryonic stages has a major impact on the in- and outflow connections of the majority of the E14.5 *Sp3*$^{-/-}$ hearts. In 15/17 E14.5 *Sp3*$^{-/-}$ hearts the aorta and the pulmonary trunk were both positioned above the right ventricle (Figure 2a,b,e,f) resulting in a double outlet right ventricle (DORV). In 2/17, solely a ventricular septal defect (VSD) was found. Although a common arterial trunk was found in four E12.5 *Sp3*$^{-/-}$ hearts, we did not find this abnormality in the E14.5 Sp3-*null* hearts analysed. However,

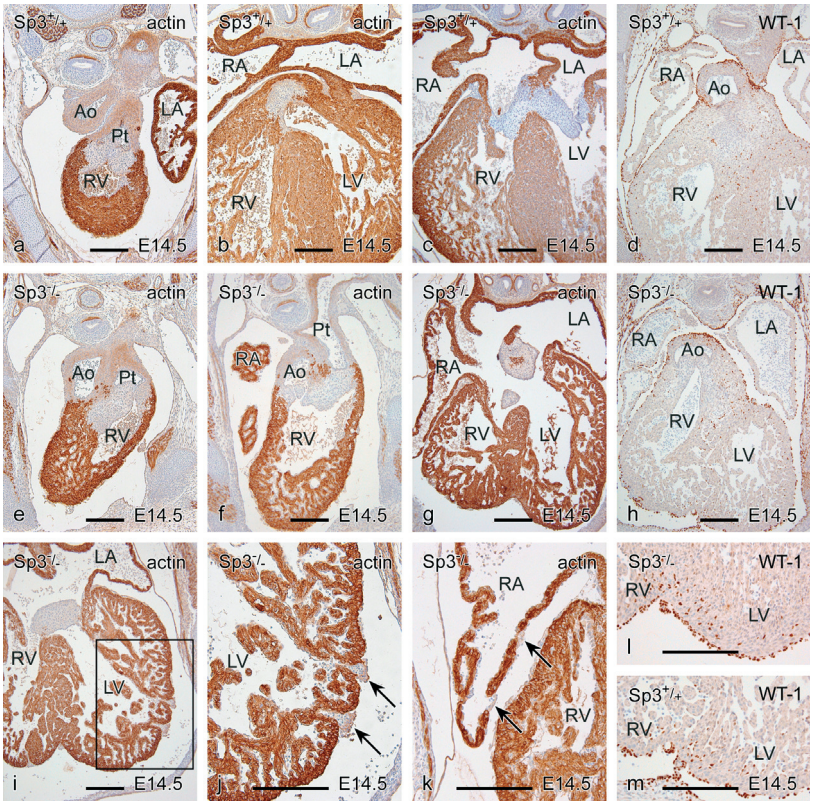


Figure 2. Histological examination of *Sp3*$^{-/-}$ hearts
(a-c, e-g, i-k) immunostaining for actin and (d,h,l,m) for WT-1, all E14.5 hearts.
The pulmonary trunk as well as the aorta connected to the right ventricle in E14.5 *Sp3*$^{-/-}$ heart (e,f), which is not the case in *Sp3*$^{+/+}$ heart (a,b). DILV malformation in E14.5 *Sp3*$^{-/-}$ heart, associated with an overriding tricuspid valve (g; *Sp3*$^{+/+}$:c). Abnormal myocardium in the Sp3 *null* mutants (i-j,k), reflected by perforated atrial and ventricular myocardium (perforations depicted by arrows in j and k), subepicardial bleeding (j) and spongy ventricular septum (compare 2c with 2i). Expression of WT-1 in the epicardial layer and EPDCs (brown dots in d,h,l,m) indicates the presence of the epicardial layer, and the invading EPDCs, in the absence of Sp3. However the epicardial layer has in irregular structure in the *Sp3*$^{-/-}$ heart (h and m). Ao: aorta; Pt: pulmonary trunk; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium. Scale bars: 100 µm.

atrio-ventricular canal malformations were found in 11/17 of the E14.5 *Sp3*$^{-/-}$ fetuses. These fetuses had a double inlet left ventricle (DILV) associated with a straddling tricuspid valve (Figure 2g,c).

Detailed examination of the *Sp3*-deficient myocardial tissue uncovered that the development of the myocardium was affected during embryogenesis. The ventricular myocardial wall of E14.5 *Sp3*$^{-/-}$ hearts was hypoplastic and had an irregular, and very spongy structure (Figure 2g,i), compared to wild-type littermates (Figure 2b,c). In addition, the ventricular septum of E14.5 *Sp3*$^{-/-}$ hearts was also composed of spongy myocardium, causing a muscular VSD in 2/17 of *Sp3*$^{-/-}$ E14.5 hearts (Figure 2g,i). Furthermore, we observed perforations in the atrial and ventricular myocardium in ~50% of the E12.5 and E14.5 *Sp3*$^{-/-}$ hearts (indicated by arrows in Figure 1f and Figure 2i-k). The perforations in the ventricular wall allowed direct sub-endocardial to sub-epicardial contact, resulting in accumulation of blood in the sub-epicardial layer.

The epicardial layer contributes to the maturation of the myocardium via the formation of epicardial derived cells (EPDCs). Absence of the epicardial layer results in the lack of

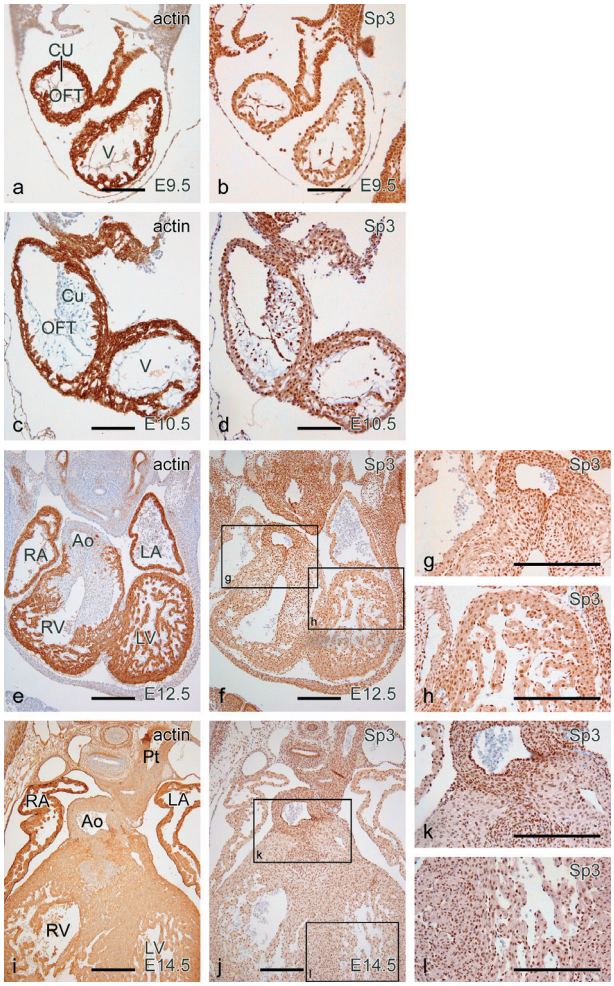


Figure 3. The expression pattern of Sp3 in E9.5-E14.5 wild-type hearts
(a,c,e,i) stained for actin and (b,d, f-h, j-l) stained for Sp3. Sp3 has a ubiquitous expression pattern in the heart as well as in the rest of the fetal body, between the developmental stages E9.5 and E14.5. (g, h) are high magnifications of f; in g the outflow region, and h the LV, of an E12.5 heart. (k, l) are high magnifications of j; in k the outflow region, and l the LV, of an E14.5 heart. In all Sp3-stained sections the main structures are positive: myocardium (b,d,f,j and magnifications); the epicardium (f,j), the mesenchymal endocardial cushion cells (d,f,g), the neural crest-derived condensed mesenchyme (asterisk in j,k) and the smooth muscle cells of the great arteries (f,g,j,k). Ao: aorta; Pt: pulmonary trunk; OFT: outflow tract; V: ventricle; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; Cu: cushion. Scale bars: 100 μm.

EPDCs and a subsequently hypoplastic compact myocardium.[19,20] The affected myocardial development in *Sp3*-deficient hearts prompted us to examine the histology of the epicardium. We made use of the expression pattern of the the Wilms' tumor gene *(WT-1*) (Figure 2d,h,l,m) as WT-1 is expressed in epicardial cells and EPDCs.[20,21] We found that there is epicardial outgrowth in the mutant hearts. However, at E12.5 the epicardial layer is abnormally attached to the myocardium and forms balloon-like structures. At stage E14.5 the number of cells in the epicardial layer was reduced, but EPDCs had invaded the myocardium of *Sp3*$^{-/-}$ hearts (Figure 2l,m).

In summary, the morphological investigation of the *Sp3*$^{-/-}$ embryonic hearts has revealed that these hearts display malformations during early cardiac formation, which develop into severe malformations at later time points.

Expression analysis

First, we analysed the Sp3 expression pattern during murine heart development at the embryonic stages E9.5-E14.5. This analysis revealed that Sp3 is ubiquitously expressed in the developing embryonic mouse heart. Thus, the myocardium, the endocardial cushion mesenchyme, the epicardium, the neural crest-derived condensed mesenchyme, and the smooth muscle cells of the great arteries that also have a neural crest origin, all express Sp3 (Figure 3b,d,f-h,j-l). To begin to understand the role of Sp3 in cardiac development, we therefore analysed expression of a set of genes known to be essential for heart development. For this purpose, we performed immunohistochemistry and radioactive in situ hybridisation experiments. The *Nkx2-5* gene is the first marker of mesodermal cells fated to cardiac development.[22,23] Nkx2-5 regulates, like Gata4, -5, and -6, many structural cardiac muscle genes.[24-26] Analysis of Nkx2-5 and Gata4 expression in E10.5, E12.5, and E14.5 Sp3-*null* hearts showed normal expression patterns of these genes (Figure 4a,e,i,m for Nkx2-5 expression and Figure 4b,f,j,n for Gata4 expression, at E12.5 and E14.5). We further analysed Tbx5, a factor essential for atrial and ventricular septation,[27] and a regulator of several cardiac genes.[28,29] The expression pattern of Tbx5 also appeared to be normal in the *Sp3*$^{-/-}$ hearts (Tbx5 expression in E12.5 hearts is shown in Figure 4d,h). We next analysed the expression of two genes known to be expressed in proliferating- and developing myocardium: *atrial natriuretic factor* (*Anf*)[30] and *cardiac ankyrin repeat protein* (*Carp*).[31,32] The expression pattern of Anf appears to be normal in the *Sp3*$^{-/-}$ hearts (Figure 4l,p shows the expression at E14.5). However, the analysis of the Carp transcript at stages E10.5-E14.5 revealed aberrant down-rgulation of Carp expression in *Sp3* mutant hearts from E12.5 onwards. At these stages, Carp expression is diminished in the compact ventricular myocardium (compare wild-type in Figure 4c with *Sp3*$^{-/-}$ in Figure 4g). At E14.5, Carp expression is lost in almost the entire *Sp3*$^{-/-}$ heart (Figure 4o). The only areas where Carp is still expressed are the right and left atrium, and part of the myocardium of the ventricular septum correlating with the position of the future conduction system.

The *iroquois related homeobox 4* gene (*Irx4*) plays a major role in specification of the heart into an atrial and ventricular section. Irx4 regulates the chamber-specific myosin isoforms by activating the *ventricular myosin heavy chain 1* gene (*Vmhc1*), and inhibiting

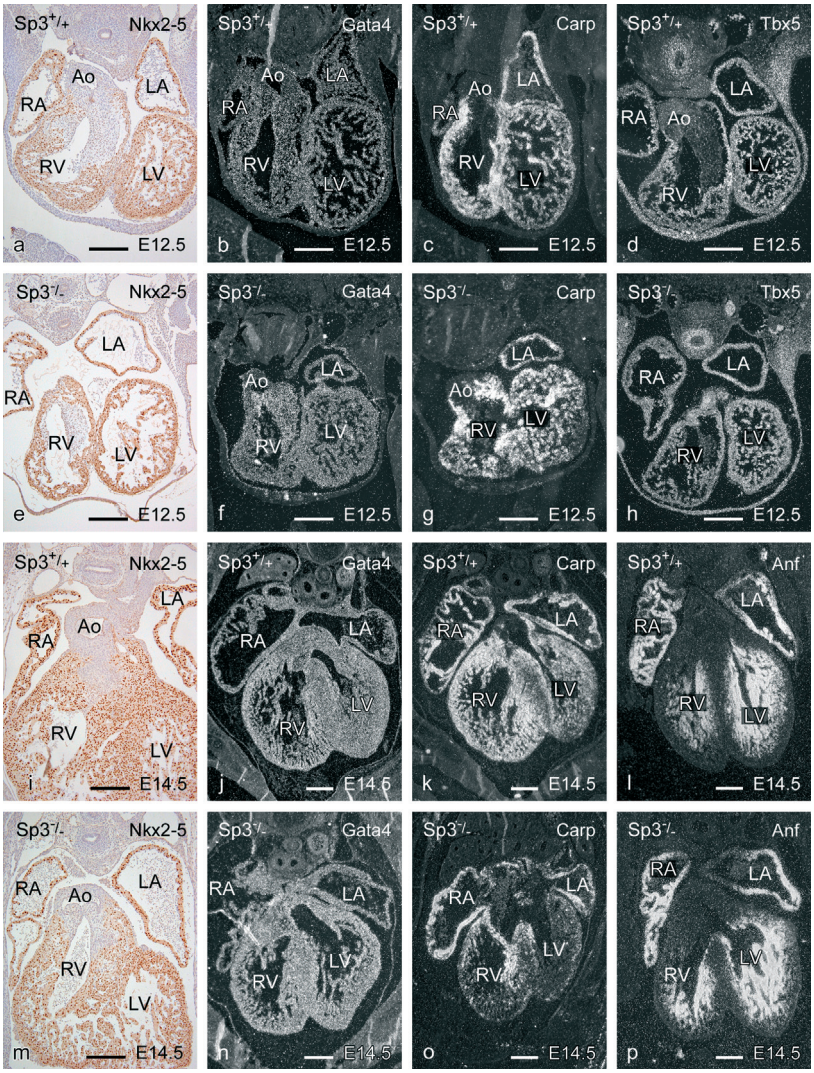


Figure 4. Expression of main regulators of cardiac development and of myocardial-specific genes

(a,e and i,m) Nkx2-5 remains expressed in the myocardium of E12.5 and E14.5 *Sp3*$^{-/-}$ hearts, (b,f and j,n) as is the case for for Gata4. (d,h) The transcript of Tbx5 has a similar pattern in the *Sp3*$^{-/-}$ as in the *Sp3*$^{+/+}$ heart at E12.5. (l,p) In the E14.5 Sp3-*null* mutant hearts, the expression pattern of Anf is indistinguishable from that in the wild-type. (c,g and k,o) Carp is down-rgulated from E12.5 in the absence of Sp3. At E12.5, Carp expression is diminished in the LV; at E14.5, Carp expression is almost completely absent in the *Sp3*$^{-/-}$ heart. The remaining expression site at E14.5 correlates with the position of the future atrio-ventricular conduction system bridging the septum. Ao: aorta; Pt: pulmonary trunk; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium. Scale bars: 100 µm.

the *atrial myosin heavy chain 1* gene (*Amhc1*) in the ventricles.[33] The in situ hybridisation analysis of Irx4 expression in E12.5 *Sp3*$^{-/-}$ hearts revealed a normal ventricular expression pattern (Figure 4a,d). Markers for correct specification of the atria and the ventricles after the primitive heart stages are the myosin light chain genes *myosin light chain 2 atrial* (*Mlc2a*) and *myosin light chain 2 ventricular* (*Mlc2v*). During early heart development the Mlc2a transcript is located in the entire heart, but it becomes restricted to the atria from E12.5 onwards,[34] whereas the *Mlc2v* gene is expressed only in the ventricular segment of the heart.[35,36] Our in situ hybridisation data show that *Sp3*$^{-/-}$ hearts have a normal Mlc2a and Mlc2v transcript distribution at developmental stages E10.5, E12.5, and E14.5 (Figure 5b,e, and c,f show the expression of Mlc2v and Mlc2a, respectively, at E12.5.)

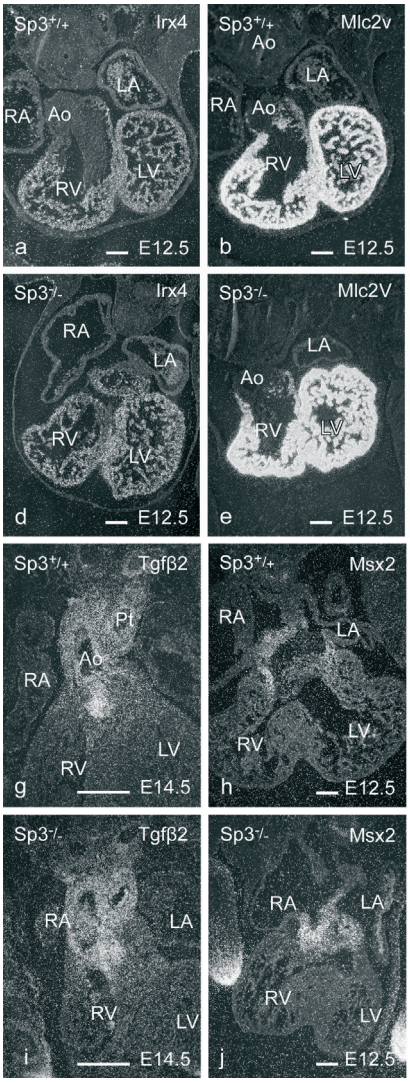


Figure 5. Analysis of expression patterns of cardiac genes by situ hybridisation
Irx4 (a,d), a mediator of ventricular-atrial specification, has normal ventricle-restricted expression in the E12.5 *Sp3*$^{-/-}$ heart. Two other markers for this specification, Mlc2V (b,e) and Mlc2A (c,f), are also normally expressed in *Sp3*$^{-/-}$ E12.5 hearts. Expression of Msx2 (h,k) and Tgfβ2 (g,i) are not altered in E12.5 and E14.5 *Sp3*$^{-/-}$ hearts. Ao: aorta; Pt: pulmonary trunk; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium. Scale bars: 100 μm.

Besides internal signals from the myocardium, the myocardium also needs signalling from the epicardium, for example via vascular endothelial growth factor (Vegf)[37] or WT-1. WT-1 is essential for outgrowth of the epicardium and is expressed in the epicardial layer and in EPDCs.[21,38] We analysed WT-1 expression in E10.5, E12.5, and E14.5 *Sp3*$^{-/-}$ hearts and found that the epicardial cells and EPDCs are expressing WT-1 normally at all stages analysed (Figure 2d,h,l,m displays WT-1 at E14.5). However, the number of EPDCs appears to be reduced in the *Sp3* null mutants.

Homeo box, msh-like 2 (Msx2) is expressed in neural crest cells and cardiac neural crest-derived mesenchyme, and is linked to formation of valves and the atrio-ventricular junction.[39] The in situ hybridisation data show that the Msx2 transcript has a normal expression pattern in E12.5 *Sp3*-deficient hearts (Figure 5h,j).

Two mouse models with heart defects similar to those observed in the *Sp3*$^{-/-}$ mouse are the *transforming growth factor* β 2 (*Tgfβ2*)[40] and *retinoic x receptor α* (*Rxrα*)[41] knockout models. This prompted us to investigate the expression of Tgfβ2 and of the two other Tgfβ members, Tgfβ1 and Tgfβ3. We observed normal expression patterns of Tgfβ1, -2, and -3 in developing *Sp3*$^{-/-}$ hearts (Tgfβ2 expression at E14.5 is shown in Figure 5 g,i). Preliminary data on in situ hybridisation with a probe for Rxrα also indicate that the expression pattern of Rxrα is normal in *Sp3*$^{-/-}$ hearts.

In summary examination of the Sp3 protein distribution unveiled the ubiquitous expression pattern of Sp3 in the developing murine heart. Analyses of the expression pattern of a panel of cardiac genes showed that most of them have a normal distribution in the *Sp3*-deficient hearts, with *Carp* as exception. Carp expression is greatly reduced in E14.5 *Sp3*$^{-/-}$ hearts. An earlier report on *Carp* positioned this gene downstream of Nkx2-5.[31] Since Nkx2-5 expression is not affected in the Sp3-*null* hearts, this suggests that Nkx2-5 mediates the expression of Carp in conjunction with Sp3.

Sp3 regulates/binds to the *Carp* promoter

The *Carp* gene is expressed in skeletal muscle, but its predominant expression site is the myocardial compartment of the heart, where it is found as early as E8. The Carp protein contains four ankyrin repeats in its carboxyl terminal end, and is nuclear localized. Carp is thought to function as a transcriptional cofactor, since in vitro experiments have demonstrated that Carp acts as an inhibitor of the expression of several cardiac genes, including *Mlc2v* and *Anf*.[31,32] Carp expression itself is mediated by Gata4, via a Gata4 site at -250 upstream of the transcription start site, and by Nkx2-5, that acts via interaction with Gata4 at the protein level.[42] However, the expression patterns of Nkx2-5 and Gata4 are not altered in *Sp3*$^{-/-}$ hearts, suggesting that Sp3 might act downstream of Nkx2-5 and Gata4 as a direct regulator of Carp transcription. We therefore searched in the 2.5 kb upstream promoter region of the *Carp* gene for GC- and GT-boxes. This region was previously shown to be necessary to drive correct Carp expression.[42] We found that this regulatory region contains five putative Sp binding sites at positions -146 (Sp-C1), -2365 (Sp-C2), -1741(Sp-C3), -1863 (Sp-C4), and -1904 (Sp-C5)

(Figure 6a). To test if these putative Sp binding sites are functional, we performed gel mobility shift assays. The sequences of the oligonucleotides used in these assays are underlined in Figure 6a. The specificity of binding was tested through the addition of a 50-fold molar excess of a competitor oligonucleotide containing two Sp binding sites.[43] The proteins present in the complexes were analysed by supershifts with specific antibodies (Figure 6b). The results show that four of the five putative Sp binding sites are capable of binding Sp3 and Sp1; only Sp-C1 is not able to bind Sp3 or Sp1 in this assay (lane 1-4 of Figure 6b). The newly identified Sp binding sites in the *Carp* promoter are flanked by two putative Nkx2-5 DNA binding sites (TNNAGTG),[44] and two putative Gata DNA binding sites (A/T GATA A/G).[45]

In summary, these experiments show that Sp3 has the capacity to bind to the Sp bindings sites present in the 2.5 upstream region of the *Carp* promoter, supporting the idea that Sp3 directly regulates the *Carp* gene.

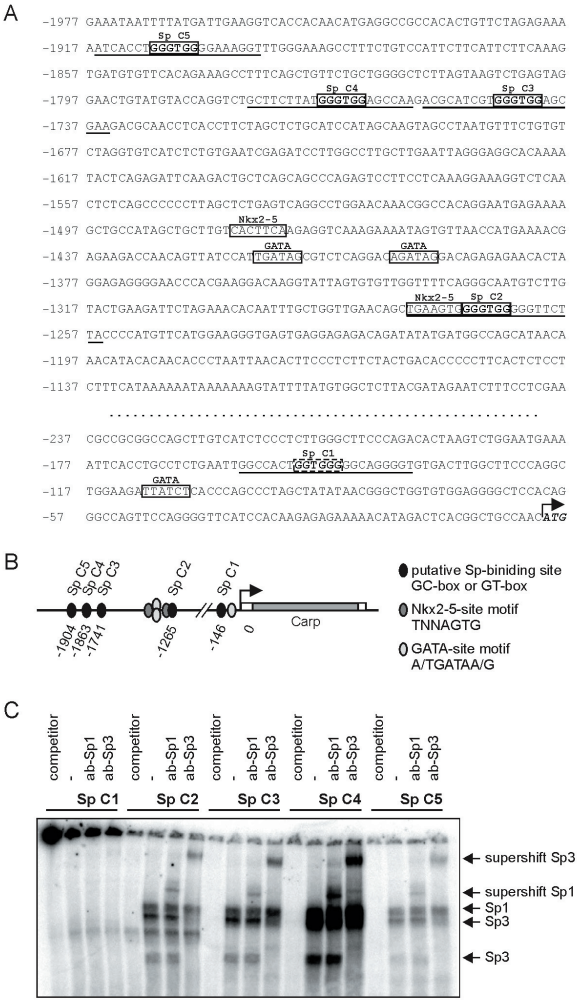


Figure 6. Functional Sp bindings sites in the murine *Carp* promoter
(a) The 2.5 kb *Carp* promoter region. The five Sp motifs are boxed (Sp C1-5), as well as the Nkx2-5 and GATA motifs. Ooligonucleotide sequences of the Sp motifs that were analysed by the gel retardation assay are underlined. (b) Schematic representation of the promoter region of the *Carp* gene. The shaded area of the Carp gene represents the translated region. (c) Gel retardation assay performed to analyse the capacity of Sp3 and Sp1 to bind to the Sp sites. The specificity of binding to the Sp C1-5 oligonucleotides was analysed through the addition of 50-fold molar excess of a competitor oligonucleotide containing two Sp sites.[43] Supershifts were performed to analyse the protein complexes with antibodies directed against Sp1 or Sp3. The arrows on the right point at the different protein complexes formed; note that there are several isoforms of Sp3.[56]

Discussion

Previous studies on Sp3$^{-/-}$ mice failed to identify the cause of perinatal mortality.[4] The new observation of a high in utero mortality rate in the C57Bl/6 background initiated this investigation of cardiac development in Sp3-*null* fetuses.

Severe cardiac malformations

The present study revealed that secondary cardiac looping is affected in *Sp3*-deficient hearts, resulting in the development of a wide inner curvature. Remodelling of the primitive heart through looping is absolutely necessary to properly establish the definitive ventricular-arterial connection. It is therefore most likely that the observed DOLV and DILV malformations, present in E14.5 *Sp3*$^{-/-}$ hearts, are brought about by disturbed looping. The observed hypoplastic and perforated compact myocardium in the *Sp3*$^{-/-}$ hearts indicates that transcription factor Sp3 is also essential for myocardial maturation. We conclude that these severe cardiac malformations are very likely causing the prenatal and perinatal death of *Sp3*$^{-/-}$ fetuses.

The *Sp3* knockout versus other knockout mice

Other knockout models display cardiac malformations comparable to those found in the *Sp3* knockout, e.g. *retinoic x receptor α* (*Rxrα*),[46,47] transforming growth factor β2 (*Tgfβ2*),[40] and friend of Gata2 (*Fog2*)[48] mutants. The *Rxrα*$^{-/-}$ mouse has in- and outflow abnormalities and hypoplastic myocardium. The E13.5 *Rxrα*$^{-/-}$ heart has elevated Mlc2a[49] and Tgfβ2[41] levels, and the Carp level is dramatically reduced from E10.5.[50] The Mlc2a and Tgfβ2 levels are normal in *Sp3*$^{-/-}$ hearts whereas the reduction in Carp expression levels follows a different pattern in *Sp3*$^{-/-}$ fetuses. Preliminary data on compound heterozygous *Sp3*$^{+/-}$:*Rxrα*$^{+/-}$ mice showed that double heterozygous fetuses do not display a significant phenotype. These observations together indicate that Rxrα and Sp3 contribute in distinct ways to development of the heart. The *Tgfβ2*$^{-/-}$ fetuses share the DORV and DILV malformations with the *Sp3*$^{-/-}$ fetuses,[40] but the Tgfβ2 transcript distribution is not altered in *Sp3*$^{-/-}$ hearts, indicating that there is no direct link between Sp3 and Tgfβ2.

Hypoplastic compact myocardium is also found in the *WT-1* knockout mouse and is caused by disturbed formation of the epicardial layer, resulting in the absence of EPDC formation in the ventricles.[21,38] Presence of EPDCs is essential for development of the compact myocardium.[19,20] *Sp3* knockout fetuses develop an epicardial layer, but the layer is not well adhered and contains fewer and irregularly distributed epicardial cells at stage E14.5, compared to the wild-type situation. We have shown previously that cardiac malformations and hypoplastic myocardium develop if attendance of EPDCs is disturbed.[19,51] We propose that the abnormal differentiation of the compact myocardium layer in *Sp3*$^{-/-}$ fetuses is the result of inadequate cross-talk between the abnormal myocardium and normal EPDCs, resulting in a phenotype that is also found after primary EPDC disturbance. This idea is supported by the normal WT-1 expression in the epicardial layer and EPDCs of *Sp3*$^{-/-}$ fetuses, despite the fact that Sp binding sites are present in the WT-1 promoter.[52]

_Sp3_-deficiency affects expression of the _Carp_ gene

Simultaneous appearance of the myocardial phenotype around E12.5 with disappearance of Carp expression suggests a direct relation between both events. Limited knowledge is available on the biological role of Carp. Carp displays negative transcriptional regulator activity on cardiac muscles genes[31] and is up-regulated during cardiac hypertrophy.[42,53] Thus, Carp might be essential for myocardial growth, a process that is impaired in the *Sp3*$^{-/-}$ hearts. However, the disordered myocardial development is not the result of Carp reduction only, since Carp is still abundantly expressed in the myocardium of the right atrium, which is not properly developed and contains perforations. We conclude that other, as yet unidentified, factors are also involved in the myocardial phenotype of *Sp3*-deficient hearts.

Carp transcription is mediated by the cooperative action of Gata4 and Nkx2-5.[42] Their normal distribution pattern in the developing *Sp3*$^{-/-}$ heart indicates that the reduction of Carp is not the result of loss of either Nkx2-5 or Gata4 expression. Our identification of four functional Sp binding sites in the 2.5 kb upstream regulatory region of the *Carp* gene, a region essential for Carp expression,[42] supports the idea that *Carp* is a direct target of Sp3-mediated transcription. However, we cannot rule out that the reduction of Carp is a secondary effect of Sp3 deficiency.

The role of Sp3 in cardiac development

Sp3-deficient embryos develop severe cardiac malformations. An intriguing question is where Sp3 is positioned in the cardiac developmental pathway. We analysed a panel of genes, including major modulators of cardiac development, and compared the Sp3 phenotype with other similar knockout phenotypes. Both approaches did shed light on the position of Sp3 in the cardiac pathway. The severe phenotype and the early wide inner curvature in the *Sp3*-deficient embryos point at Sp3 as one of the crucial factors in cardiac development downstream of Nkx2-5. This is underlined by an experiment with chimeric embryos in which wild-type blastocysts have been injected with Sp3-*null* ES cells. The chimeric embryos were harvested at developmental stage E14.5 and examined by serial sectioning. Most of these embryos had developed severe cardiac malformations similar to those found in the normal *Sp3*$^{-/-}$ embryos. Even embryos with a relatively low contribution of *Sp3*$^{-/-}$ cells to the heart and other mesodermal structures developed severe malformations, including DORV and hypoplastic compact myocardium. Thus, unlike the *Rxrα* knockout,[54,55] the phenotype of the *Sp3* knockout appears to be cell autonomous. This chimeric experiment, together with our other observations, indicates Sp3 as a crucial mediator in heart development.

Further characterization of the Sp3 heart phenotype is necessary for accurate positioning of Sp3 in the pathway of cardiac development. Since Sp3 is a transcription factor we would like to identify more direct downstream target genes of Sp3. Furthermore, as Sp3 is ubiquitously expressed, the *Sp3* knockout phenotype can be the result of improper functioning of several cell types (such as epicardial- and neural crest cells) that interact with heart development. A conditional knockout strategy should help to reveal which cell types contribute to the spectrum of the *Sp3*$^{-/-}$ heart phenotype.

Materials and methods

Mice

Sp3 knockout mice[4] were bred for more than 10 generations to C57Bl/6 mice. Embryos were derived from timed breedings of *Sp3*$^{+/-}$ x *Sp3*$^{+/-}$ mice. Genotyping was performed by polymerase chain reaction (PCR), with the use of 3 primers: a sense primer in the *Sp3* gene amplifying the wild-type allele (5'-GCGTGCAAGCCAGTGGTC-3'), a sense primer in the *Neo* gene amplifying the knockout allele (5'-AGCGCATCGCCTTCTATCG-3'), and a common antisense primer in the *Sp3* gene (5'-GGACGATTCTATGATGCCTCC-3'). PCR conditions are 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds for 30 cycles.

In situ hybridisation

Probes for Tgfβ1, Tgfβ2, and Tgfβ3 have been described;[15] the probe for Mlc2v was a gift from A.F. Moorman. Other probe sequences were generated via PCR and subsequently cloned using the pGEM-T-Easy vector system I (Promega, A1360), and sequenced. Riboprobes were labeled with ^{35}S-UTP (Amersham, SJ603, 1000 Ci/mmol) and hybridisation was performed as described previously.[16] See Table 1 for the PCR primers that were used to generate the probes.

Immunostaining

E9.5-E14.5 embryos were immersion-fixed in a 4% para-formaldehyde (PFA) solution in PBS at 4°C overnight, E16.5 fetuses were first perfused with the 4% PFA solution via the liver, and then immersion-fixed in PFA at 4°C overnight. The embryos were embedded in paraffin and serially sectioned (5 µm) transversely. The following antibodies were used. First antibodies: HHF35 (Dako, M365); Mlc2a (S.W. Kubalak); Nkx2-5 (Santa Cruz Biotechnology, sc-8697); Sp3 (Santa Cruz Biotechnology, sc-13018); WT-1 (Santa Cruz Biotechnology, sc-192). Secondary antibodies: biotinylated goat anti rabbit (Vector Laboratories BA-1000) for Mlc2a, Sp3 and WT-1; biotinylated rabbit anti goat (Vector Laboratories, PK-6105) for Nkx2-5; and for HHF35 rabbit anti mouse (Dako, P0260), goat anti rabbit (Nordic Immunological Laboratories,

Table 1. PCR primers used to generate the probes

gene symbol	primers	fragmet size (bp)
Anf	5'- GACAGCAAACATCAGATCGT-3' (s) 5'-CTCTGGGCTCCAATCCTGTC-3' (as)	461
Carp	5'-AGGAGCTGGTAACAGGCAAA-3' (s) 5'-TTCAGGACATCTGCGTTTCC-3' (as)	489
Gata4	5'-AATGCCTGTGGCCTCTATCA-3' (s) 5'-CGCTGATTACGCGGTGATTA-3' (as)	621
Gata5	5'-GACTTTGCCTTCACCTCCT-3' (s) 5'-AGTCCTGCGTCTGTAAGCAA-3' (as)	541
Irx4	5'-ATGCTGGCAAAGACGACAAG-3' (s) 5'-GGTGGCCCAGGCCTGGTTCA-3' (as)	623
Mlc2a	5'-GCACAACGTGGCTCTTCTAA-3' (s) 5'-GTGGGTGATGATGTAGCAGA-3' (as)	455
Tbx5	5'-AAGACACCTTCTATCGCTCG-3' (s) 5'-TATTCTCACTCCACTCTGGC-3' (as)	504

Gar-Ig), and Rabbit-peroxidase anti peroxidase (Nordic Immunological Laboratories, R-PAP). The Vectastain ABC kit HRP (Vector Laboratories PK6100) was used as the third step for biotinylated secondary antibodies. For visualization, sections were incubated with 400 mg/ml DAB (3-3'diaminobenzidine tetrahydrochloride, Sigma-Aldrich Chemie D5637) in 0.05 mM Tris-maleate buffer pH 7.6, and finally counterstained with Mayer's hematoxylin.

Gel retardation assay

Gel retardation assay were performed as described.[17] The sequences of the oligonucleotides used are (the sense strand is given):

C1 5'-ACCCCTGCCCCCACCAGTGGC-3'
C2 5'-TAAGAACCCCCACCCCACTTCA-3'
C3 5'-TTCGCTCCACCCACGATGCGT-3'
C4 5'-TTGGCTCCACCCATAAGAAGC-3'
C5 5'-ACCTTTCCCCACCCAGGTGAT-3'
Sp 5'-TTATGGGCGGAGTTAGGGGCGGGACTAT-3'

Samples were incubated for 30 min at room temperature, loaded on a 4% polyacrylamide/ 0.5×TBE gel, and ran at 250 V for 2 hours at room temperature. Polyclonal antibodies against Sp1 and Sp3 were used at 1:16 dilution in the binding reactions. Nuclear protein isolation was performed as described.[18]

References

1. Philipsen, S. & Suske, G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* **27**, 2991-3000 (1999).
2. Bouwman, P. & Philipsen, S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* **195**, 27-38 (2002).
3. Marin, M., Karis, A., Visser, P., Grosveld, F. & Philipsen, S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* **89**, 619-28 (1997).
4. Bouwman, P. et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. *Embo J* **19**, 655-61 (2000).
5. Gollner, H., Dani, C., Phillips, B., Philipsen, S. & Suske, G. Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**, 77-83 (2001).
6. Van Loo, P. F. et al. Impaired hematopoiesis in mice lacking the transcription factor Sp3. *Blood* **102**, 858-66 (2003).
7. Gollner, H. et al. Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. *Genes Cells* **6**, 689-97 (2001).
8. Supp, D. M., Witte, D. P., Branford, W. W., Smith, E. P. & Potter, S. S. Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility. *Dev Biol* **176**, 284-99 (1996).
9. Nguyen-Tran, V. T. et al. A novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages. *Cell* **102**, 671-82 (2000).
10. Brandon, E. P., Idzerda, R. L. & McKnight, G. S. Targeting the mouse genome: a compendium of knockouts (Part III). *Curr Biol* **5**, 873-81 (1995).
11. Brandon, E. P., Idzerda, R. L. & McKnight, G. S. Targeting the mouse genome: a compendium of knockouts (Part II). *Curr Biol* **5**, 758-65 (1995).
12. Brandon, E. P., Idzerda, R. L. & McKnight, G. S. Knockouts. Targeting the mouse genome: a compendium of knockouts (Part I). *Curr Biol* **5**, 625-34 (1995).
13. Fishman, M. C. & Chien, K. R. Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-117 (1997).
14. Cripps, R. M. & Olson, E. N. Control of cardiac development by an evolutionarily conserved transcriptional network. *Dev Biol* **246**, 14-28 (2002).
15. Molin, D. G. et al. Expression patterns of Tgfbeta1-3 associate with myocardialisation of the outflow tract and the development of the epicardium and the fibrous heart skeleton. *Dev Dyn* **227**, 431-44 (2003).
16. Hierck, B. P., Poelmann, R. E., van Iperen, L., Brouwer, A. & Gittenberger-de Groot, A. C. Differential expression of alpha-6 and other subunits of laminin binding integrins during development of the murine heart. *Dev Dyn* **206**, 100-11 (1996).

17. Wall, L., deBoer, E. & Grosveld, F. The human beta-globin gene 3' enhancer contains multiple binding sites for an erythroid-specific protein. *Genes Dev* **2**, 1089-100 (1988).
18. Andrews, N. C. & Faller, D. V. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* **19**, 2499 (1991).
19. Gittenberger-de Groot, A. C., Vrancken Peeters, M. P., Bergwerff, M., Mentink, M. M. & Poelmann, R. E. Epicardial outgrowth inhibition leads to compensatory mesothelial outflow tract collar and abnormal cardiac septation and coronary formation. *Circ Res* **87**, 969-71 (2000).
20. Gittenberger-de Groot, A. C., Vrancken Peeters, M. P., Mentink, M. M., Gourdie, R. G. & Poelmann, R. E. Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ Res* **82**, 1043-52 (1998).
21. Moore, A. W., McInnes, L., Kreidberg, J., Hastie, N. D. & Schedl, A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. *Development* **126**, 1845-57 (1999).
22. Azpiazu, N. & Frasch, M. tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. *Genes Dev* **7**, 1325-40 (1993).
23. Bodmer, R. The gene tinman is required for specification of the heart and visceral muscles in Drosophila. *Development* **118**, 719-29 (1993).
24. Lee, Y. et al. The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol Cell Biol* **18**, 3120-9 (1998).
25. Lien, C. L. et al. Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development* **126**, 75-84 (1999).
26. Small, E. M. & Krieg, P. A. Transgenic analysis of the atrialnatriuretic factor (ANF) promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of ANF. *Dev Biol* **261**, 116-31 (2003).
27. Garg, V. et al. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424**, 443-7 (2003).
28. Bruneau, B. G. et al. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106**, 709-21 (2001).
29. Hiroi, Y. et al. Tbx5 associates with Nkx2-5 and synergistically promotes cardiomyocyte differentiation. *Nat Genet* **28**, 276-80 (2001).
30. Houweling, A. C., Somi, S., Van Den Hoff, M. J., Moorman, A. F. & Christoffels, V. M. Developmental pattern of ANF gene expression reveals a strict localization of cardiac chamber formation in chicken. *Anat Rec* **266**, 93-102 (2002).
31. Zou, Y. et al. CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway. *Development* **124**, 793-804 (1997).
32. Jeyaseelan, R. et al. A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. *J Biol Chem* **272**, 22800-8 (1997).
33. Bao, Z. Z., Bruneau, B. G., Seidman, J. G., Seidman, C. E. & Cepko, C. L. Regulation of chamber-specific gene expression in the developing heart by Irx4. *Science* **283**, 1161-4 (1999).
34. Kubalak, S. W., Miller-Hance, W. C., O'Brien, T. X., Dyson, E. & Chien, K. R. Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *J Biol Chem* **269**, 16961-70 (1994).
35. Lee, K. J. et al. Myosin light chain-2 luciferase transgenic mice reveal distinct regulatory programs for cardiac and skeletal muscle-specific expression of a single contractile protein gene. *J Biol Chem* **267**, 15875-85 (1992).
36. O'Brien, T. X., Lee, K. J. & Chien, K. R. Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc Natl Acad Sci U S A* **90**, 5157-61 (1993).
37. Tomanek, R. J., Ratajska, A., Kitten, G. T., Yue, X. & Sandra, A. Vascular endothelial growth factor expression coincides with coronary vasculogenesis and angiogenesis. *Dev Dyn* **215**, 54-61 (1999).
38. Kreidberg, J. A. et al. WT-1 is required for early kidney development. *Cell* **74**, 679-91 (1993).
39. Abdelwahid, E., Rice, D., Pelliniemi, L. J. & Jokinen, E. Overlapping and differential localization of Bmp-2, Bmp-4, Msx-2 and apoptosis in the endocardial cushion and adjacent tissues of the developing mouse heart. *Cell Tissue Res* **305**, 67-78 (2001).
40. Bartram, U. et al. Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta(2)-knockout mice. *Circulation* **103**, 2745-52 (2001).
41. Kubalak, S. W., Hutson, D. R., Scott, K. K. & Shannon, R. A. Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos. *Development* **129**, 733-46 (2002).
42. Kuo, H. et al. Control of segmental expression of the cardiac-restricted ankyrin repeat protein gene by distinct regulatory pathways in murine cardiogenesis. *Development* **126**, 4223-34 (1999).
43. Gillemans, N. et al. Altered DNA-binding specificity mutants of EKLF and Sp1 show that EKLF is an activator of the beta-globin locus control region in vivo. *Genes Dev* **12**, 2863-73 (1998).
44. Chen, C. Y. & Schwartz, R. J. Identification of novel DNA binding targets and regulatory domains of a murine tinman homeodomain factor, nkx-2.5. *J Biol Chem* **270**, 15628-33 (1995).
45. Whyatt, D. J., deBoer, E. & Grosveld, F. The two zinc finger-like domains of GATA-1 have different DNA binding specificities. *Embo J* **12**, 4993-5005 (1993).

46. Kastner, P. et al. Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003 (1994).
47. Gruber, P. J. et al. RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J Clin Invest* **98**, 1332-43 (1996).
48. Tevosian, S. G. et al. FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101**, 729-39 (2000).
49. Dyson, E. et al. Atrial-like phenotype is associated with embryonic ventricular failure in retinoid X receptor alpha -/- mice. *Proc Natl Acad Sci U S A* **92**, 7386-90 (1995).
50. Ruiz-Lozano, P. et al. Energy deprivation and a deficiency in downstream metabolic target genes during the onset of embryonic heart failure in RXRalpha-/- embryos. *Development* **125**, 533-44 (1998).
51. Lie-Venema, H. et al. Ets-1 and Ets-2 transcription factors are essential for normal coronary and myocardial development in chicken embryos. *Circ Res* **92**, 749-56 (2003).
52. Cohen, H. T., Bossone, S. A., Zhu, G., McDonald, G. A. & Sukhatme, V. P. Sp1 is a critical regulator of the Wilms' tumor-1 gene. *J Biol Chem* **272**, 2901-13 (1997).
53. Redfern, C. H. et al. Conditional expression of a Gi-coupled receptor causes ventricular conduction delay and a lethal cardiomyopathy. *Proc Natl Acad Sci U S A* **97**, 4826-31 (2000).
54. Chen, J., Kubalak, S. W. & Chien, K. R. Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development* **125**, 1943-9 (1998).
55. Tran, C. M. & Sucov, H. M. The RXRalpha gene functions in a non-cell-autonomous manner during mouse cardiac morphogenesis. *Development* **125**, 1951-6 (1998).
56. Sapetschnig, A., Koch, F., Rischitor, G., Mennenga, T. & Suske, G. Complexity of translationally controlled transcription factor Sp3 isoform expression. *J Biol Chem* (2004).

CHAPTER 4

Chapter 4

Identification of target genes of the transcription factor Sp3 in fetal murine heart by microarray experiments

Pieter Fokko van Loo, Joost Broekhuis, Jun Hou, Frank Grosveld, and Sjaak Philipsen

Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands

Abstract

Murine Sp3-*null* embryos die from serious cardiac dysfunction that is brought about by severe cardiac abnormalities occurring during fetal heart development. This shows taht transcription factor Sp3 is an essential transcriptional mediator in the developmental process that gives rise to the murine heart. However, the biological role of Sp3 in cardiac development remains largely unsolved since only one putative cardiac target gene of Sp3 has been identified, the *Carp* gene. To search for candidate target genes of Sp3, we performed microarray experiments and compared the expression patterns of E12.5 and E14.5 murine *Sp3*$^{-/-}$ hearts to wild-type hearts of the same developmental stages. Two-colour microarray experiments were executed on arrays representing 23,000 murine genes with the hybridization of experimental RNA against a reference RNA. This provides a reference signal on each array that enables the comparison of individual arrays to the entire data set. The data images were normalized with the LOESS procedure at first, and were subsequently analyzed by a one-way ANOVA procedure and clustering analysis. Despite some technical shortcomings, the one-way ANOVA procedure identified eleven genes that are down-regulated in E12.5 and E14.5 *Sp3*$^{-/-}$ hearts. Six of these transcripts are potentially involved in cardiac development, and five out of these six sequences are designated as candidate target genes of Sp3 on the basis of *in silico* promoter analysis

Introduction

The ubiquitously expressed transcription factor specificity protein 3 (Sp3) is essential for life, as mice lacking this factor are severely growth retarded and succumb to perinatal death.[1] The Sp3 protein has been implicated in late tooth development of fetuses as well as skeletal ossification.[1,2] Furthermore, Sp3 has a role in the hematopoietic system, since Sp3 deficiency causes cell autonomous defects in the erythroid and myeloid lineages.[3] To unravel the cause of early lethality, lung development was first examined. This investigation revealed only minor morphological alterations in the lungs of embryonic day 18.5 (E18.5) *Sp3*$^{-/-}$ embryos, alterations of a type that are most probably not severe enough to cause immediate post-partum death.[1] More recently we found that the early lethality is most likely caused by severe cardiac malformations. The cardiac malformations of the *Sp3*$^{-/-}$ embryo include imperfect in- and outflow connections of the heart as well as hypoplastic myocardium. These cardiac malformations collectively result in a serious dysfunctional heart during late embryogenesis (van Loo et al., submitted for publication), explaining the observed lethality of the *Sp3* knockout phenotype.

Sp3 belongs to the Sp/XKLF (specificity protein/x Krüppel-like factor) family, which consists of at least 25 transcription factors (for reviews see references [4-6]). The Sp/XKLF factors share three conserved Krüppel-like Cys_2-Hys_2 zinc fingers, located C-terminally. These fingers form the DNA binding domain, recognizing the GC-(GGGGCGGGG) box and the related GT-(GGGGTGTGG) box,[7-10] abundantly represented in the proximal and distal regulatory regions of housekeeping- and tissue-specific genes. The Sp subclass of the family comprehends nine Sp-factors. Two of these, Sp1 and Sp4, share a high degree of similarity to Sp3. They have similar zinc finger regions that bind with the same affinity to the GC- and GT-boxes as well as similar glutamine-rich activation domains in their N-terminus.[6] Nevertheless, each factor has distinct biological functions, as exemplified by the phenotypes of Sp1-, Sp3-, and Sp4-*null* mice. Mice deficient for Sp1, the founding member of the Sp/XKLF family,[11] are severely growth retarded and die around E10.5.[12] In contrast, *Sp4*$^{-/-}$ mice are born alive displaying an initial growth retardation. They are infertile and display e increased early postnatal mortality possibly caused by cardiac arrhythmia,[13-15]

The Sp3 protein fulfils a role as transcriptional mediator via binding to GC- and GT-boxes in the regulatory elements of its target genes. As a mediator of transcription, Sp3 has two faces: it is either an activator or a repressor of transcription. Which face Sp3 will manifest, depends on (1) the composition of the target regulatory elements: Sp3 activates promoters with a single Sp-binding site, but fails to do so with promoters with multiple adjacent Sp-binding sites;[5,16,17] (2) the isoform composition: the long isoforms of Sp3 can activate transcription from the SV40 promoter (Simian virus 40), whereas the short Sp3 isoforms cannot;[18] (3) the post-transcriptional modification: upon SUMOylation, Sp3 is completely transcriptionally silenced.[19,20]

To fully understand the function of Sp3 we need to identify its target genes. So far, only a couple of putative target genes of Sp3 have been identified during the analysis of the phenotype of Sp3$^{-/-}$ embryos: the ameloblast-specific a*meloblastin* gene and the cardiac-specific and the c*ardiac ankyrin repeat protein* (Carp) gene. The Ameloblastin transcript, which is involved in enamel formation of the tooth,[21,22] is down-regulated in the ameloblast layer of E18.5 *Sp3*$^{-/-}$ embryos and its promoter region contains putative bindings sites for Sp3.[1] The *Carp* gene, which is essential for the development of the myocardium, is down-regulated in E14.5 *Sp3*$^{-/-}$ hearts and four Sp bindings site are present in the promoter region of this gene (van Loo, et al., this thesis). However, the origin of all cardiac malformations that are found in Sp3-*null* fetuses cannot solely be explained by the absence of Carp expression, since the cardiac abnormalities already appear between E10.5 and E12.5. This suggests that Sp3 regulates other genes that are important for proper cardiac development. As the outcome of the previously undertaken detailed morphological examination of heart development in *Sp3*$^{-/-}$ embryos revealed severe cardiac malformation we performed microarray experiments in order to discover novel Sp3 target genes. We compared the gene expression patterns in *Sp3*$^{-/-}$ hearts with those of wild-type littermates of E12.5 and E14.5 fetuses. The data images were normalized with the LOESS (local regression) procedure,[23] and analyzed by one-way ANOVA (analysis of variance) and clustering analysis. The genes found by these analyses were scrutinized for their potential relevance to the Sp3-*null* cardiac phenotype and examined as putative target genes of Sp3 through *in silico* promoter analysis.

Results and Discussion

Experimental setup

To understand the function of Sp3 in cardiac development we aimed to discover Sp3 target genes by comparing the expression patterns of E12.5 (n=5) and E14.5 (n=5) *Sp3*$^{-/-}$ hearts to wild-type hearts of the same age by the executing of oligonucleotide microarray experiments. We conducted two-colour oligonucleotide microarray experiments, as this set-up provides a reference signal on each array when an experimental RNA sample is hybridized against a RNA sample of a reference standard RNA pool, labelled with a second fluorescent dye. Such a reference signal should allow us to compare each individual array to the entire dataset. With regards to the RNA pool, as we had experimental RNA of E12.5 and E14.5 hearts, we prepared for each stage an accompanying RNA pool. The RNA pools were composed of the combined total RNA of twenty E12.5 or E14.5 wild-type hearts. Furthermore, dye swap experiments were performed to be able to compensate the data for potential bias caused by the fluorescent dyes used. The dye swaps were otherwise executed as an exact repeat of the first hybridization. With these robust conditions in mind, we carried out forty experimental hybridizations and four control hybridizations (Table 1). Due to human error, we did not obtain results for one Sp3$^{-/-}$-Cy3 versus pool E12.5-Cy5 microarray hybridization.

	Cy3 dye	Cy5 dye
E12.5	*Sp3*$^{+/+}$ (n=5)	pool E12.5
	Sp3$^{-/-}$ (n=5)	pool E12.5
	pool E12.5	*Sp3*$^{+/+}$ (n=5)
	pool E12.5	*Sp3*$^{-/-}$ (n=5)
E14.5	*Sp3*$^{+/+}$ (n=5)	pool E14.5
	Sp3$^{-/-}$ (n=5)	pool E14.5
	pool E14.5	*Sp3*$^{+/+}$ (n=5)
	pool E14.5	*Sp3*$^{-/-}$ (n=5)
controls	pool E12.5 (n=1)	pool E12.5
	pool E12.5 (n=1)	pool E14.5
	pool E14.5 (n=1)	pool E12.5
	pool E14.5 (n=1)	pool E14.5

Table 1. Experimental set-up of two-colour microarray experiments with Cy3 and Cy5 dyes
E12.5 and E14.5 experimental samples were hybridized against E12.5 and E14.5 reference pool RNA, composed of twenty RNA samples of E12.5 or E14.5 wild-type hearts, respectively. The number of hybridized arrays per experiment and controls are indicated between brackets.

The amount of messenger RNA (mRNA) that can be obtained from E12.5 and E14.5 hearts is not sufficient to execute dye swap experiments for each experimental sample if direct labelling is used. This forced us to amplify the mRNA by T7-based RNA-amplification. Next, the labelling of the aaRNA was executed by covalent binding of a fluorescent dye to aminoallyl-UTP that was incorporated during the RNA amplification step. This post-amplification RNA-labelling procedure is particularly convenient for dye swap experiments. The hybridization and washing of the arrays was automated, and arrays containing a 23K mouse oligonucleotide library were used.

Analysis of the arrays

Spot intensity. The scan files of the hybridized arrays were first analyzed with the Imagene software to quantify the fore- and background intensity of each spot, and to flag the empty and bad spots. The flagging procedure flagged between 37% and 73% of the spots on the individual arrays, with an average of 55% per array. Next, the data were normalized to adjust the microarray data for effects that are a result of variation in the technology rather than from biological differences between the RNA samples, to obtain unbiased and relative expression for each spot. The normalization was done with the LOESS procedure, a statistic feature to perform local adjustment on non-parametric data,[24] and by utilizing the entire grid of the array. The normalized data were subsequently analyzed with Rosetta Resolver software.

Dye swap experiments. Normally, dye swap experiments should provide similar data, but with opposite polarity as the fluorescent dyes have been swapped between the experiments. When they contain similar data, the dye swap experiments will pair, and thereby the significance of the final outcome of the microarray experiments will increase. Unfortunately, the pairing analysis of our dye swap experiments revealed that these did not pair, indicating a technical problem. On most of the arrays, many spots (on average 55%) had been flagged. The lack in pairing might therefore be caused by the large number of flagged spots. When the raw microarray data (before flagging and normalization) was used for pairing analysis, the

dye swap experiments did not pair either. Taken together, the lack of the dye swaps to pair indicates that these microarray data have limited consistency between technical replicates. For the further analysis of the data, the dye-swap arrays were considered as two individual experiments, but with opposite polarities.

ANOVA analysis. Despite the limited consistency between the experiments, the examination of the data was continued by ANOVA statistics analysis.[25] Anova statistics assesses if there is a significant difference between the averages of the groups, despite the variance within the groups, and gives a single P-value to the null hypothesis that there is no difference. The high variance between the individual *Sp3*$^{-/-}$ experiments made it impossible to employ two-way ANOVA analysis for the identification of the statistically significant changes in gene expression between wild-type and *Sp3*$^{-/-}$ hearts. Therefore, we performed one-way ANOVA analysis, and compared individual E12.5 or E14.5 *Sp3*$^{-/-}$ experiments with the E12.5 or E14.5 wild-type dataset group. Both dataset groups were comprised of all wild-type experiments of E12.5 or E14.5, respectively, and P<=0.05 was set as the criterion for differential expression of a gene. This basis gave per individual *Sp3*$^{-/-}$ experiment a list of genes that were differentially expressed with a certainty of 95% in the particular *Sp3*$^{-/-}$ experiment. By charting those sequences that were differentially expressed in at least two or three individual *Sp3*$^{-/-}$ experiments per developmental stage, we enhanced the significance of these listed sequences. These charting lists show that 284 and 50 genes are differentially expressed in at least two and three E12.5 *Sp3*$^{-/-}$ experiments, respectively, whereas that 209 and 42 genes are differentially expressed in at least two and three E14.5 *Sp3*$^{-/-}$ hearts, respectively. From those listed genes, eleven genes are represented in the lists with 50 and 42 genes, the E12.5- and E14.5-shortlist. These eleven genes are represented in Table 2.

The one-way ANOVA analysis and the subsequent charting has supplied four lists of genes that are differentially expressed with P<=0.05 in at least two and three E12.5 or E14.5

gene symbol	gene description
Tpk1	thiamin pyrophosphokinase 1
Spock1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1
Ypel1	yippee-like 1
Defcr-rs12	defensin related cryptin, related sequence 12
Abca8b	ATP-binding cassette, sub-family A 8b
Lcn5	lipocalin 5
Slc6a20	solute carrier family 6 member 20 (or Xtrp3A)
Abdh1	alpha/beta hydrolase domain containing 1
Nhlh2	nescient helix-loop-helix 2
1810029E06	10 day old male pancreas cDNA, clone:1810029E06
Gb33r	immunoglobulin light chain variable region gb33r pseudogene

Table 2. Eleven differentially expressed transcript
One-way Anova analysis identified eleven murine transcripts that are differentially expressed in at least three *Sp3*$^{-/-}$ E12.5 as well as three *Sp3*$^{-/-}$ E14.5 microarray experiments in comparison to the wild-type dataset group, with P<=0.05. Shown are the current sequence symbol/name, sequence description and the accession number of representative sequences.

Sp3$^{-/-}$ hearts. However, as the one-way ANOVA analysis only aims to identify a significant difference between two groups, it did not provide any insight in the fold-change in expression of these genes. So, it is possible that a listed gene is up regulated in one E12.5 experiment, while it is down-regulated in the other two E12.5 experiments.

Because the discovery of these genes is the most significant result of the analysis, as they are differentially expressed at two developmental stages, we concentrated on the eleven genes (Table 2) for further examination.

Clustering analysis. It is important to know if these eleven sequences, although assigned as differentially expressed at the E12.5 and E14.5 stages, are up or down-regulated in the individual *Sp3*$^{-/-}$ experiments. But above all, is it essential to know if the direction of the changed expression (up- or down-regulated) level is similar between all individual *Sp3*$^{-/-}$ experiments at both developmental stages, as a similar change might be expected between both developmental stages. Because the one-way ANOVA analysis did not give information on changes in expression levels, we performed a clustering analysis. A clustering analysis gives, besides a representation of the spot intensity per gene and per array, a clustering of the arrays that display a comparable expression pattern of those genes on which the clustering analysis is performed as well as a clustering of those genes by their relative fold changes. We performed clustering analysis of the arrays in a two-dimensional clustering by the fold change expression of the genes listed in the two shorts list (n=50 for E12.5 and n=42 for E14.5) that were generated by the ANOVA analysis. Hierarchical clustering, a non-supervised clustering analysis, clustered eight out of the eleven previously selected sequences together with a high correlation coefficient in the direction of the fold-changes (Figure 1, horizontal direction), namely Abca8b, Abdh1, Defcr-rs12, Lcn5, Spock1, Slc6a20, Tpk1, and Ypel1. The Gb33r and Nhlh2 genes clustered together in this direction with a coefficient of ~0.8. In the vertical direction, this clustering displays no direct correlation between arrays of the same the genotype (Figure 1). Even more concerning is that the arrays with Cy5 labelled experimental RNA (called Cy5-arrays) were almost separated from the arrays with the Cy3 labelled experimental RNA (called Cy3-arrays). An additional performed clustering analysis on the basis of the expression pattern of these genes that are listed in the two long lists (n=284 for E12.5 and n=209 for E14.5) shows even a more dramatic clustering of the Cy5-arrays away from the Cy3-arrays (data not shown). Furthermore, the clustering analysis shown in Figure 1 reveals that the arrays had the tendency to form a subcluster per developmental stage, beyond clustering per fluorescent dye. In a K-means supervised clustering, with the condition that there are two (E12.5 and E14.5) or four (*Sp3*$^{-/-}$ and *Sp3*$^{+/+}$ of both stages) vertical groups, the vertical clustering did not improve either. Additional hierarchical and K-means clustering analysis with only the Cy5- or Cy3-arrays did also not improve the clustering of the *Sp3*$^{+/+}$ arrays from the *Sp3*$^{-/-}$ arrays.

That the clustering analysis has clustered eight out of the eleven selected sequences together by their relative fold change in expression is of no value, as the experiments/arrays cluster by the dye used and not by their genotype in the vertical dimension. So the outcome of cluster analysis did not reveal that the selected eleven sequences have a similar direction in

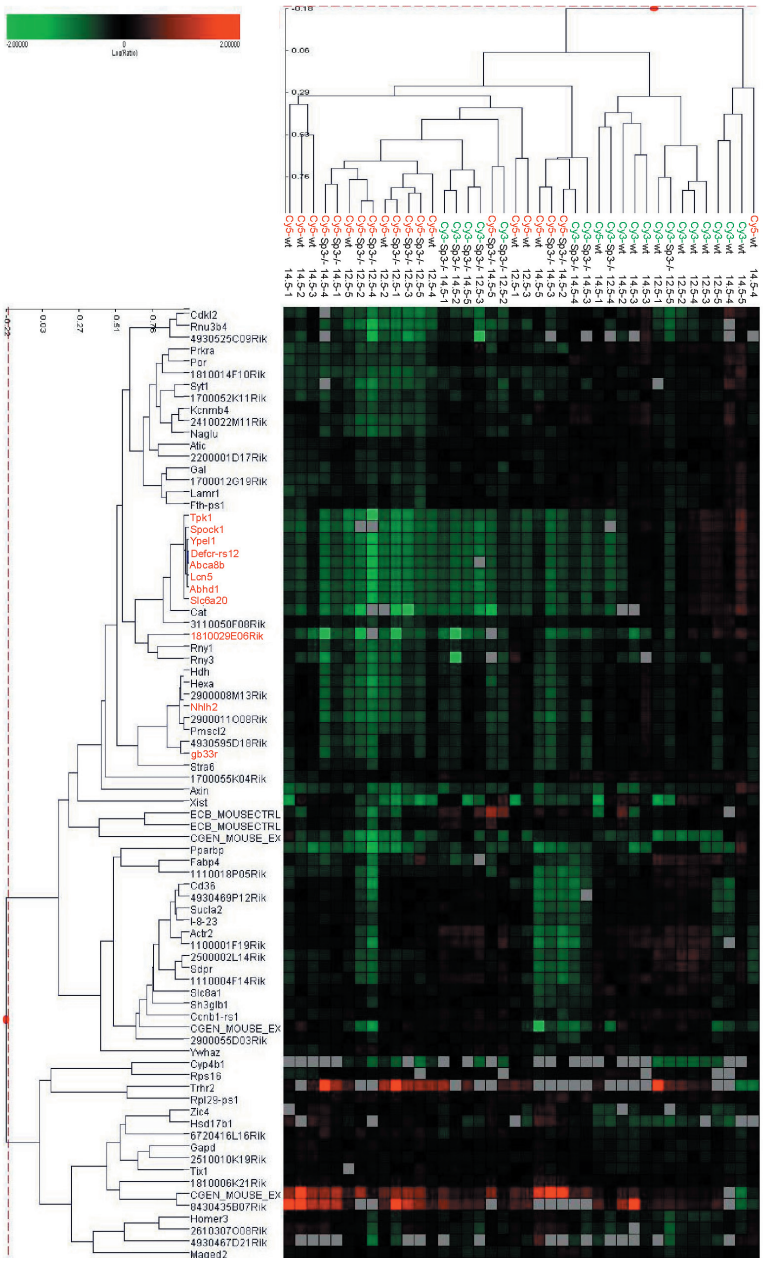


Figure 1. Clustering analysis of the microarrays

Hierarchical clustering analysis of all microarrays by the fold change of gene expression for the gene listed in the two long lists generated by the ANOVA analysis (n=284 for E12.5 and n=209 for E14.5). From left to right, depicted clustering of the genes by their fold changes, degree of clustering is indicated by correlation coefficient from 0 to 1, and sequence names. From top to bottom clustering, is depicted degree of clustering of the arrays. Genotype, age and fluorescent dye of the experiment as indicated. Red spot reflects a higher signal in the Cy5-channel over Cy3-channel, green spot reflects higher Cy3-signal over Cy5-signal; brighter spot reflects larger difference between two fluorescent dyes; black spot reflects an equal Cy3- and Cy5-signal; grey spot stands for a flagged spot.

the change of their expression in the E12.5 and E14.5 Sp3-*null* experiments. The tendency of the arrays to subcluster by developmental stage could be an indication that not many genes are differentially expressed in the *Sp3*$^{-/-}$ experiments.

Whereas the observed clustering of the Cy3-arrays away from the Cy5-arrays underlines the earlier statement that our microarray experiments have a serious limitation in the consistency of the data and suggests that this is caused by a technical shortcoming at the level of the RNA-labelling procedure. We used identical aaRNA samples as starting material to perform the labelling of the dye-swap experiments. Therefore, the technical shortcoming has to be searched at the level of covalent binding of the fluorescent dyes to the aminoallyl-RNA, influence of the fluorescent dyes used on the hybridization or at the level of the detection of the signal. The clustering analyses also show that the clustering method is a fundamentally different method to the one-way ANOVA procedure. The advantage of the ANOVA procedure is that it has the capacity to search for significant differences between groups with a certain P-value, in spite of the presence of variance within the groups. This makes this analysis method very suitable and valuable for the analysis of our microarray data.

Fold changes in expression. In a final attempt to know how the expression of the eleven genes in the *Sp3*$^{-/-}$ experiments has changed relative to the wild-type experiments, we compared the Cy5/Cy3-ratio of the *Sp3*$^{-/-}$ experiments with those of the *Sp3*$^{+/+}$ experiments. This ratio, calculated by division of the spot intensity in Cy5-channel by the spot intensity in the Cy3-channel, is positive when the Cy5-signal higher is than the Cy3-signal. When the Cy3-signal is higher than the Cy5-signal, the ratio is negative, as the ratio than is a recalculated by the division -1/ratio. For the comparison of the ratios we classified the ratios-values above +15 and below -15 as an unreliable outcome of the experiment, and therefore we excluded them from the comparison. Furthermore, the ratios of the Cy3-experiments were inversed to compensate for the polarity difference between the Cy5- and Cy3-experiments,

	E12.5			E14.5		
gene symbol	Cy5-exp	Cy3-exp	average	Cy5-exp	Cy3-exp	average
Tpk1	-3.3	-4.1	-3.7	-2.9	-3.1	-2.6
Spock1	-0.7	-5.5	-3.1	-2.4	-3.9	-3.1
Ypel1	-2.9	-4.7	-3.8	-2.0	-3.3	-2.6
Defcr-rs12	-5.7	-4.6	-5.2	-2.0	-3.4	-2.7
Abca8b	-2.3	-4.1	-3.2	-2.2	-3.5	-2.9
Lcn5	-2.2	-4.9	-3.6	-2.4	-2.8	-2.6
Slc6a20	-1.7	-4.1	-2.9	-2.4	-3.5	-2.9
Abdh1	-2.7	-5.2	-4.0	-2.2	-3.6	-2.9
Nhlh2	-2.6	-0.7	-1.6	-2.3	-2.1	-2.2
1810029E06	-1.5	-3.6	-2.5	-2.3	-2.7	-2.5
Gb33r	-2.2	-0.5	-1.3	-1.6	-2.2	-1.9

Table 3. Fold changes in expression of the eleven transcripts
Average fold-change expression of the eleven selected sequence, relative to the reference signal, by comparison of the ratios of the Cy5/Cy3 spot intensities and represented as a Cy5 labelled experimental RNA. Normalized Cy5/Cy3 ratios were averaged per gene, developmental stage, genotype and fluorescent dye and subsequently wild-type ratio was subtracted from the accompanying *Sp3*$^{-/-}$ ratio.

thereby Cy3-experiments were represented as Cy5-experiments. The Cy5/Cy3-ratios were averaged per gene, genotype, and developmental stage as well as per fluorescent dye used for labelling of the experimental RNA, as the previous clustering analysis revealed the huge difference between the experiments of the Cy3 and Cy5 labelled RNA. Subsequently, the averages of the *Sp3*$^{+/+}$ experiments were subtracted from the accompanying averages of the *Sp3*$^{-/-}$ experiments. The subtracted Cy5/Cy3-ratios were negative for all the eleven genes per developmental stage and per fluorescent dye used (Table 3), indicating that they are truly down-regulated in the *Sp3* knockout hearts of both developmental stages. Along with the outcome of the averaged and subtracted ratios, the Cy5/Cy3-ratios of the eleven transcripts on each individual *Sp3*$^{-/-}$ array have all the same direction change in expression in comparison to the reference signal. Nevertheless, the value of the examination is limited, because it is based on averaged ratios and does not take the variance in the wild-type group and the individual Sp3-*null* experiments into account. The combination of this examination together with the one-way ANOVA analysis, which does take the variance within a group into account, makes the statement that these genes are down-regulated in E12.5 and E14.5 Sp3-*null* hearts most likely.

In summary, by the combination of one-way ANOVA, clustering, and examination of the average fold change we have identified eleven sequences that have the tendency to be down-regulated in E12.5 and E14.5 *Sp3*$^{-/-}$ hearts. However, as the microarray data have a limited consistency, these results have to be validated in more detail.

Are the eleven genes linked with Sp3?

An intriguing question is if these eleven discovered genes, although assigned as differentially expressed at the E12.5 and E14.5 stages of the *Sp3*-deficient embryos, have a functional link with the cardiac phenotype of the *Sp3* knockout mice. This question will be addressed by a discussion on known the biological characteristics of each gene, in alphabetic order. The genes that by this assessment are regarded to have a putative relation with the Sp3-*null* model will next be examined as possible candidate target genes of Sp3, by an *in silico* analysis of their promoter regions for the presence of putative GC-, GT-boxes, and CACCC-boxes, which are the binding motifs of Krüppel-like factors.[7-10,26] The promoter regions of the human and rat homology of these genes will be implicated in this *in silico* analysis, when available.

Abca8b. This gene belong to the A subfamily of ATP-binding cassette (ABC) transporter family. The proteins from this family share two nucleotide binding folds (NBF) with conserved Walker A and B motifs, a signature sequence and are localized in the cellular membrane by two membrane spanning domains (MSD1 and 2).[27-29] They translocate a wide variety of substances, including ions, peptides, sugars, vitamins, and steroid hormones, across cellular membranes by hydrolysis of ATP.[30-32] The *Abca8b* gene is expressed during embryogenesis and in the neonatal heart and in most other organs.[33] Abca8b is suggested to be a drug translocator, as the human Abca8 protein can translocate drugs across the cellular membrane.[33,34] The

presence of one GGTGG- and two GGTGGG-motifs at positions -409, -853, and -1237 in the 5'-flanking region of the murine *Abca8b* gene as well as at similar positions in the 5'-flanking region of the rat *Abca8b* gene, do suggest that Sp3 potentially regulates the expression of the *Abca8b* gene. However, a role as drug transporter during heart development is highly unlikely. It may fulfill a more versatile role in transmembrane transport than only transport of drugs, as the ABC proteins have been implicated in the transport of variety of molecules.[30-32]

Abdh1. The *alpha/beta hydrolase domain containing 1* gene has a N-terminal located transmembrane domain, an α/β hydrolase fold with unknown function, and it belongs to the prosite UPF0017 alpha/beta hydrolase protein family.[35,36] The gene transcript is found during embryogenesis, and has also an abundant expression pattern in neonatal mice, including the heart.[35] The 500 bp upstream region of the murine *Abdh1* gene is highly conserved between mouse and rat, and is rich of conserved GC- and GT-boxes. A single GT-box is located at position -464 (in murine locus), while conserved individual and clustered GC- and GT-boxes are found in the murine and rat upstream regions between -1 and -150 bp (Figure 2a). The 5'-flanking region of human *Abdh1* gene harbours as well many GC- and GT-boxes (Figure 2b), but this sequence does not align with the corresponding murine and rat 5'-flanking region. The presence of the putative GC- and GT-boxes in these three upstream regions, postulates *Abdh1* as a candidate target gene of Sp3. However, one can only speculate on a role of the Abdh1 protein in the cardiac malformations of the *Sp3*$^{-/-}$ fetuses, as there are no functional data on the Abdh1 protein and on the α/β hydrolase fold available.

Defcr-rs12. Cryptin is a defensin-related peptide, expressed in the epithelial cells of intestinal crypts,[37] which belongs to a large family of antimicrobial molecules.[38,39] A link of an antimicrobial protein with the *Sp3* knockout phenotype appears far-fetched. We therefore regard this sequence as noise in our microarray experiments.

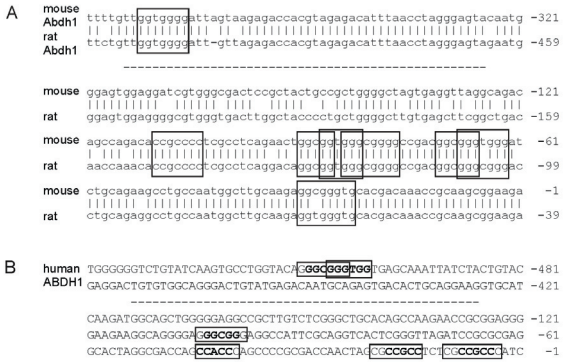


Figure 2. Analysis of the *Abdh1* promoter region
(a) Alignment of the 400 bp 5-prime upstream region of the murine and rat *Abdh1* genes displays the conservation of the single and clustered GC- and GT-boxes sequences between mouse and rat. GC- and GT-boxes are in the murine region present at -27, -63, -69, -81, -85, -87, -107, and -369. (b) The 500 bp region 5-prime upstream the transcription initiation site of human *Abdh1* gene harbours putative GC- and GT-box at the positions -4, -15, -41, -101, -502, and -506. The region does not align with corresponding of mouse and rat 5' upstream regions. GC- and GT-boxes marked by rectangles.

Gb33r. This sequence is a pseudogene of an immunoglobulin κ-light chain variable region.[40] This sequence has to be classified as irrelevant for our current research.

Lcn5. Lcn5 (lipocalin 5) was identified as an androgen-dependent protein in the rat epididymis that is excreted in the lumen of the epididymis and binds retinoic acid.[41-43] While many reports found expression of *Lcn5* gene solely in the epididymis by Nothern blot analysis,[43,44] Zwain and co workers found immuno-reactive material present in testis, prostate, seminal vesicles, liver, spleen, and brain of 22, 60, and 120 days old rats (the heart was not examined).[45] However, as they detected signals in these organs of approximately 1% of the signal of the epididymis, these observations may be due to aspecific signals, thereby leaving the discussion on the expression pattern open. The function of this lipocalin protein is elusive as well, as the retinoic acid binding capacity was assumed solely by an in vitro assay.[41,42] Nevertheless, more retinoic acid binding lipocalin proteins are found, for example lipocalin-type prostaglandin D synthase (Lpgds)[46,47] and bovine beta-lactoglobulin (Blg).[48] Vitamin A has also been shown to be essential for cardiac development. For example, the *retinoic x receptor α* (*Rxrα)* knockout mouse displays a cardiac phenotype similar to our *Sp3* knockout model.[49,50] Furthermore, CACCC-boxes are present in the 550 bp 5'-flanking region of the *Lcn5* gene at positions -76, -115, -178, and -248; a GC-box is found at position -351. Of these boxes, the CACC-boxes are conserved in the rat homolog of the murine *Lcn5* gene, *epididymal secretory protein 1* (*Esp1*) gene.[44] The presence of the CACCC- and GC-boxes in the 5'-flanking region of murine *Lcn5*, along with its putative function as retinoic acid transporter and the vital role of retinoic acid in heart development, postulate *Lcn5* as a putative target gene for Sp3, although the question of its expression pattern has to be solved yet.

Nhlh2. This basic helix-loop-helix (blhl) transcription factor is predominantly expressed in the nervous system, but is absent in fetal and neonatal heart.[51,52] *Nhlh2* knockout studies have revealed that the Nhlh2 protein is associated with adult onset of obesity and disturbed sexual behaviour.[53,54] The *Nhlh2*$^{-/-}$ phenotype together with the absence of Nhlh2 expression in the heart suggest that the identification of this gene by our microarray experiments is not informative.

Spock1. Spock1 is a proteoglycan protein, with structural domains that are related to molecules that have been associated with adhesion, migration, and cell proliferation.[55] The abundant expression of its transcript in the synaptic fields of the developing nervous system during embryogenesis, and at adulthood in the brain and Schwann cells[56-59] suggest that Spock1 fulfils a role in neurogenesis. This points out that this gene is most likely not associated with the *Sp3*$^{-/-}$ cardiac phenotype, although a role in the cardiac conduction system can not be ruled out.

Slc6a20. The gene has been assigned to the solute carrier family 6, which belongs to the plasma membrane neurotransmitter transporter superfamily. Member of this superfamily transport neurotransmitter molecules into the cell against the concentration gradient, by energy derived from the co-transport of Na^+/Cl^-.[60,61] The assignment of Slc6a20 to the solute carrier family 6 was based on its cDNA sequence, but not on functional analysis of the protein. Expression analysis in mouse and human revealed that Slc6a20 is only expressed in the

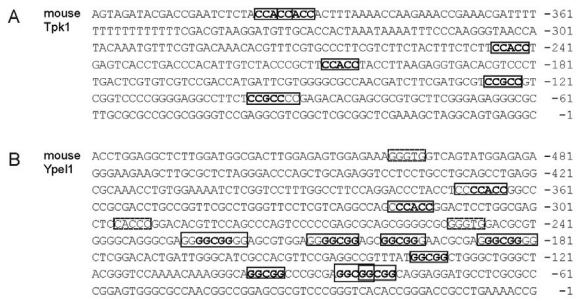


Figure 3. Analysis of the promoter regions of the *Tpk1* and *Ypel1* genes
(a) GC- and GT-box sequences are present in the 400 bp upstream region of *Tpk1* gene at positions -95, -123, -205, -242, -392, and -394. (b) The 5'-flanking region of the murine *Ypel1* gene contains GC- and GT-boxes at positions -81, -83, -95, -133, -183, -197, -204, -222, -315, and -365 as well as CACCC-boxes at positions -248, -293, and -496. GC- and GT-boxes marked by rectangles with continuous line, CACC-boxes marked by rectangles with dashed line.

kidney.[60] The absence of Slc6a20 expression in the heart makes it highly unlikely that this gene is involved in *Sp3* knockout phenotype: this sequence has to be regarded as noise in our microarray experiment.

Tpk1. This enzyme catalyzes the conversion of thiamin (vitamin B_1) into thiamin pyrophosphate (or TTP)[62] and facilitates thiamin transport into cell.[63,64] The TTP molecule is an essential biological cofactor in carbohydrate metabolism, including the Krebs cycle,[65,66] and therefore is Tpk1 an essential protein. A shortage of thiamin or TTP causes the Beriberi syndrome in human (displaying cardiovascular and neurological defects)[67] or the more severe neuropathological Wernicke-Korsakoff syndrome.[68] Expression analysis of the *Tpk1* gene mapped its transcript to a variety of mouse organs, including the heart.[69] Murine Tpk1 expression is possibly mediated by Sp3, as several putative GC- and GT-boxes are present in the 500 bp upstream of its transcription initiation site (Figure 3a), although these appear to be weak Sp binding sites. In addition, these sites are not conserved between mouse and human, but the first 500 bp of the human *Tpk1* gene do harbour GC-, GT-, and CACCC-boxes as well. There is no data on a direct correlation of thiamin, TTP or Tkp1 with heart development. Despite this, the putative role of Tpk1 in the *Sp3* knockout model has to be studied, as the heart expresses Tpk1 and thiamin is vital for the carbohydrate metabolism and energy supply.

Ypel1. Examination of the Ypel1 expression at E9.5 and E10.5 revealed that it is expressed in the ventral mesoderm at E9.5 and at E10.5 in the branchial clefts, the branchial arches and the atria, and truncus arteriosus of developing heart.[70] Upon transfection in fibroblasts, this nuclear protein seems to induce an epithelial-like transition accompanied with the cytoskeleton and cell adhesion machineries.[70] The 500 bp of the murine *Ypel1* gene upstream region harbours three CACCC-boxes as well as ten putative GC- or GT-boxes (Figure 3b). The corresponding human region is also rich of potential GC- and GT-boxes, but does not align to the murine region. Collectively, *Ypel1* might be a true Sp3 target gene but a possible role in heart development remains to be established.

1810029E06Rik. This E10 mouse male pancreas EST is obtained from a high efficiency full-length cDNA cloning effort.[71] No functional studies have been performed on this sequence.

In summary, although all the eleven sequence have not been directly linked with cardiac development, six of these sequences, namely Abca8b, Abdh1, Lcn5, Tpk1, Ypel1, and 1810029E06Rik have the potential to be involved in cardiac development and are candidate target genes of Sp3. To examine if these sequences are *in vivo* Sp3 target genes, the value of the found putative GC-, GT-, and CACCC-boxes in their promoter regions has to be evaluated by gel mobility assays and chromatin immune precipitation (ChiP) assays.[72] Besides this, as the data on the biological function of these genes is too limited to speculate on a link between these six sequences and the *Sp3*$^{-/-}$ cardiac phenotype, the biological processes that are depending on these putative Sp3 the target genes have to be disclosed well as the biochemical characteristics of these genes have to be examined in detail. Furthermore, the fact that these six sequences have been found to be differentially expressed in E12.5 as well as E14.5 *Sp3*$^{-/-}$ hearts increases the significance of the discovery of the six sequences. However, the down-regulation of the *Abca8b, Abdh1, Lcn5, Tpk1,* and *Ypel1* genes as well as 1810029E06Rik EST can also be due to secondary effects of Sp3 deficiency.

Concluding remarks

Previous research on the *Sp3*$^{-/-}$ cardiac phenotype revealed Carp as a putative Sp3 target gene by in situ hybridisation and analysis of the Carp promoter (van Loo et al., this thesis). Carp is down-regulated in the ventricles and the left atria of E14.5 *Sp3*$^{-/-}$ hearts and its promoter regions harbours canonical Sp binding sites. In contrast to these experiments, the analysis of our microarray experiments on E12.5 and E14.5 *Sp3*$^{-/-}$ hearts did not mark *Carp* as a differentially expressed gene. The absence of Carp in the list of differentially expressed genes is not caused by a low signal of the Carp spot on the arrays, as the Carp spot was positive on the arrays that were hybridized with both wild-type and *Sp3*$^{-/-}$ experimental RNA. To elicit which technology gives the correct result for Carp expression, Carp expression should be determined by additional techniques, for instance by Nothern blot analysis. However, the technical shortcomings that we have encountered in our microarray exponents argue in favour of the in situ hybridization data.

The absence of the notification of Carp as a differentially expressed gene in the *Sp3*$^{-/-}$ hearts by the microarray experiments is an additional indication that a technical shortcoming has limited the data output of the microarray analysis. All these indications argue for a repeat of these microarray experiments. However, if the experiment would be repeated, the experimental set-up of the microarray experiments may have to be adjusted, as the gene expression during the early phases of heart development *Sp3*$^{-/-}$ mice should be examined as well. A local cardiac abnormality during the primitive heart stage can bring about severe cardiac abnormalities during later cardiac development and can even cause prenatal death, as has been shown by a mouse line in which *Mlc2a* is deleted specifically in the atrium before the looping of the heart tube.[73] The lesson from this observation for our *Sp3* knockout model is that the cardiac abnormalities of the *Sp3*-null embryos may have their onset during the early phase of heart development. By our microarray analysis of the gene expression patterns at

E12.5 and E14.5 *Sp3*$^{-/-}$ hearts, we may have missed the target genes of Sp3 that cause the heart phenotype. Therefore the gene expression pattern in *Sp3*$^{-/-}$ hearts staged between the fusion of the cardiac crescent into a linear tube and the primary looping of the heart tube[74] would have to be examined.

In summary, our microarray study to discover candidate target genes of Sp3 in order to unravel the *Sp3*$^{-/-}$ cardiac phenotype has provided six transcripts that are down-regulated in E12.5 and E14.5 *Sp3*$^{-/-}$ hearts. These transcripts are potentially involved in cardiac development in cooperation with Sp3, and five of them are potential direct Sp3 target genes based on the presence of Sp binding sites in their promoters. Additional investigation on their role in the development of the *Sp3* knockout cardiac phenotype is necessary to understand the role that Sp3 fulfils in cardiac development.

Materials and methods

Mice

Sp3 knockout mice[1] were bred for more than thirteen generations to C57Bl/6 mice and embryos were derived from timed breedings of *Sp3*$^{+/-}$ x *Sp3*$^{+/-}$ mice. Genotyping was done by polymerase chain reaction (PCR), with the use of three primers: for amplifying the wild-type allele a sense primer in the *Sp3* gene (5'- GCGTGCAAGCCAGTGGTC-3'), for amplifying the knockout construct a sense primer in the *Neo* gene (5'-AGCGCATCGCCTTCTATCG-3'), and with common antisense primer in the *Sp3* gene a (5'-GGACGATTCTATGATGCCTCC-3'). PCR conditions: 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C for 30 cycles.

Microarrays

Batch D of the microarrays of by the Biomics core facility of ErasmusMC Rotterdam has been used for the experiments. These microarrays contain the 23K mouse LabOnWeb oligonucleotide library (Sigma/Compugen), representing approximately 23,000 mouse genes.

RNA isolation and preparation

RNA was isolated with TRI Reagent (Sigma T9224). The quality and the concentration of the RNA was determined with the Agilent 2100 BioAnalyzer on a RNA 6000 Nano LabChip, and the BioSpec-mini spectrophotometer, respectively.

Probe preparation

Denaturation of the RNA and the annealing of the oligonucleotide to the RNA was done by adding 3 µg total RNA in 11 µl solution of 0.155 M Trehalose (Fluka 90208) 23 ng/µl T7dt (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-dT$_{(18)}$-3') oligonucleotide, and an incubation for 10' at 65ºC followed by 5' on ice. To this mixture, RNAase guard (Amersham Biosciences 27-0185-01), dNTPs (Roche 1922505), DTT (Invitrogen 8064-071), and Superscript II reverse transcriptase (Invitrogen 18064-071) were added to a 20 µl 1x First strand buffer (Invitrogen 18064-071) mixture to an end concentration of 1.5 µU, 0.5 mM, 10 µM, and 10 U, respectively, to synthesize first strand cDNA. The incubation conditions were: 1.5 hours at 42°C, 10' at 65°C, and 5' on ice.

The second strand cDNA was synthesized by adding 30 µl 5x Second strand buffer (Invitrogen 10812-014), 30 µmol dNTPs (Roche 1922505), 40 Units *E. coli* DNA ligase (Invitrogen 18052-019), 40 Units *E.coli* DNA polymerase (Invitrogen 18010-025), and 2 U RNaseH (Promega M4285) to the first strand cDNA mixture, and finally adjusted to an end volume of 150 µl with H_20. This mixture was incubated for 2 hours at 16°C. The synthesis was completed by an addition of 2 µl T4 DNA polymerase 3 units/µl (Roche 1004786) and a subsequent incubation for 10' at 16°C.
The newly synthesized cDNA was purified by a phenol/chloroform/isoamylalcohol (Invitrogen 15593-031) extraction, and by subsequent washing and concentration of the cDNA on a micron-30 column (Clontech 636069).
RNA was amplified with and according to the Ambion Megascript T7 kit (Ambion 1334), with exception for the amount of UTP added. For UTP, we added 5-(3-aminoallyl)-UTP (Ambion 8437) together with non-modified UTP in a ratio of 3:1, with a final UTP concentration of 7.5 mM. The entire amount ofcDNA synthesized was used for RNA amplification.
Prior to the coupling of Cy-label to the aminoallylRNA (aaRNA), the aaRNA was purified and concentrated to a volume of 10 µl with microcon YM-30 column (Millipore 4241). For the coupling of Cy-label to the aaRNA, 3 µg aaRNA in 10 µl 50% DMSO (dimethyl sulfoxide)/0.03 M Na_2C0_3 pH9.0 was added to dried Cy3- or Cy5-dyes (Amersham Biosciences RPN5661) and incubated for 1 hour at room temperature in the dark. Subsequently, the probe was purified and concentrated on a microcon column YM-30 (Millipore 4241) to a final volume of approximately 30 µl.

Hybridization and scanning of arrays
To reduce the background signal, the arrays were presoaked in 2xSSC/0.05%SDS (sodium dodecyl sulfate)/0.25% Sodium borohydride (Sigma S-9125) for 20 minutes at 42ºC, and washed three times in 1xSSC for 30 seconds at room temperature. Arrays were subsequently pre-hybridized in 5xSSC/0.05%SDS/4%BSA (Bovine Serum Albumin, Roche 0711454) at 42ºC for 15 minutes, washed at room temperature for 1 minute in 1xSSC, twice for 30 seconds in 0.2xSSC, for 30 seconds with H_20, and finally dried by centrifugation at 1350 g for 2 minutes. Arrays were hybridized and washed on a Tecan Hybridization Station 4800. Prior to hybridization, the arrays were first washed two times with 2xSSC/0.05%SDS for 45 seconds at 45ºC. Probe, dissolved in 100 µl Pronto universal hybridization solution (Corning 40024), was injected at 52ºC and hybridized for 16 hours at 45ºC with medium agitation frequency. Afterwards, the arrays were washed two times for 1 minute with 2xSSC/0.05%SDS at 45ºC, four times for 45 seconds with 1xSSC at 23ºC and a soak time of 1 minute, two times for 2 minutes with 0.2xSSC at 23ºC, and for 30 seconds with 0.2xSSC at 23º and with a soak time of 30 seconds. Arrays were finally rinsed with H_20 and dried with N_2 for 2 minutes at 30ºC.
The micro arrays were scanned with the Perkin Elmer ScanArray Express HT scanner.

Data analysis
The spot intensities on the arrays were quantified with Biodiscovery's ImaGene 5.6 program, and expression values for the spots were extracted using anautomatic segmentation grid. An automated flagging scheme was used to markspots that would be excluded from the analysis, e.g. empty, negative, and poor spots.
The raw intensity files were normalized with the 'Limma' model from Bioconductor, using the method of 'LOESS (Local Regression) pin grid normalization'.[23] The landmarks, control spots, and flagged spots were not taken into account in the normalization. The background of the

entire array was corrected with 'Minimum', then any intensity that was zero or negative after background subtraction was set equal to half the minimum of the positive corrected intensities for that array.

The normalized intensity files were uploaded into Rosetta Resolver v4.0 (RosettaBio, US). One-way *ANOV*A was employed to identify the statistically significant changes in expression levels using a set of hybridizations of wild type hearts as the expected expression, and P<=0.05 was set as the criterion for differentially expressed genes. Selecting those genes that were differentially expressed in at least two or three individual experiments per developmental stage enhanced the significance of identification. To account for variation due to labelling, those genes that had no signal from both channels, and those genes for which less than a 0.05 P-value was found in expression changes between two colours in a self-self hybridization were excluded from the analysis results. The hierarchical and K-means clustering with K=2 or 4 (cluster initialization: data centroid based search, similarity measure: Pearson correlation) were performed using normalized ratios (2 log) of genes that were identified differentially expressed.

References

1. Bouwman, P. et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. *Embo J* **19**, 655-61 (2000).
2. Gollner, H., Dani, C., Phillips, B., Philipsen, S. & Suske, G. Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**, 77-83 (2001).
3. Van Loo, P. F. et al. Impaired hematopoiesis in mice lacking the transcription factor Sp3. *Blood* **102**, 858-66 (2003).
4. Bouwman, P. & Philipsen, S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* **195**, 27-38 (2002).
5. Suske, G. The Sp-family of transcription factors. *Gene* **238**, 291-300 (1999).
6. Philipsen, S. & Suske, G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* **27**, 2991-3000 (1999).
7. Gidoni, D. et al. Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. *Science* **230**, 511-7 (1985).
8. Giglioni, B., Comi, P., Ronchi, A., Mantovani, R. & Ottolenghi, S. The same nuclear proteins bind the proximal CACCC box of the human beta-globin promoter and a similar sequence in the enhancer. *Biochem Biophys Res Commun* **164**, 149-55 (1989).
9. Imataka, K. [Side effects and safety in drug therapy of hypertension]. *Nippon Rinsho* **50 Suppl**, 381-7 (1992).
10. Letovsky, J. & Dynan, W. S. Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. *Nucleic Acids Res* **17**, 2639-53 (1989).
11. Kadonaga, J. T., Carner, K. R., Masiarz, F. R. & Tjian, R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**, 1079-90 (1987).
12. Marin, M., Karis, A., Visser, P., Grosveld, F. & Philipsen, S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* **89**, 619-28 (1997).
13. Gollner, H. et al. Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. *Genes Cells* **6**, 689-97 (2001).
14. Supp, D. M., Witte, D. P., Branford, W. W., Smith, E. P. & Potter, S. S. Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility. *Dev Biol* **176**, 284-99 (1996).
15. Nguyen-Tran, V. T. et al. A novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages. *Cell* **102**, 671-82 (2000).
16. Dennig, J., Hagen, G., Beato, M. & Suske, G. Members of the Sp transcription factor family control transcription from the uteroglobin promoter. *J Biol Chem* **270**, 12737-44 (1995).
17. Birnbaum, M. J. et al. Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. *Biochemistry* **34**, 16503-8 (1995).
18. Sapetschnig, A., Koch, F., Rischitor, G., Mennenga, T. & Suske, G. Complexity of translationally controlled transcription factor Sp3 isoform expression. *J Biol Chem* (2004).
19. Ross, S., Best, J. L., Zon, L. I. & Gill, G. SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell* **10**, 831-42 (2002).

20. Sapetschnig, A. et al. Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *Embo J* **21**, 5206-15 (2002).
21. Krebsbach, P. H. et al. Full-length sequence, localization, and chromosomal mapping of ameloblastin. A novel tooth-specific gene. *J Biol Chem* **271**, 4431-5 (1996).
22. Lee, S. K. et al. Ameloblastin expression in rat incisors and human tooth germs. *Int J Dev Biol* **40**, 1141-50 (1996).
23. Mariani, T. J. et al. A variable fold change threshold determines significance for expression microarrays. *Faseb J* **17**, 321-3 (2003).
24. Smyth, G. K. & Speed, T. Normalization of cDNA microarray data. *Methods* **31**, 265-73 (2003).
25. Kerr, M. K., Martin, M. & Churchill, G. A. Analysis of variance for gene expression microarray data. *J Comput Biol* **7**, 819-37 (2000).
26. Crossley, M. et al. Isolation and characterization of the cDNA encoding BKLF/TEF-2, a major CACCC-box-binding protein in erythroid cells and selected other cells. *Mol Cell Biol* **16**, 1695-705 (1996).
27. Higgins, C. F. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**, 67-113 (1992).
28. Chang, G. & Roth, C. B. Structure of MsbA from E. coli: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science* **293**, 1793-800 (2001).
29. Locher, K. P., Lee, A. T. & Rees, D. C. The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science* **296**, 1091-8 (2002).
30. Bauer, B. E., Wolfger, H. & Kuchler, K. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim Biophys Acta* **1461**, 217-36 (1999).
31. Klein, I., Sarkadi, B. & Varadi, A. An inventory of the human ABC proteins. *Biochim Biophys Acta* **1461**, 237-62 (1999).
32. Holland, I. B. & Blight, M. A. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J Mol Biol* **293**, 381-99 (1999).
33. Annilo, T., Chen, Z. Q., Shulenin, S. & Dean, M. Evolutionary analysis of a cluster of ATP-binding cassette (ABC) genes. *Mamm Genome* **14**, 7-20 (2003).
34. Tsuruoka, S. et al. Functional analysis of ABCA8, a new drug transporter. *Biochem Biophys Res Commun* **298**, 41-5 (2002).
35. Edgar, A. J. & Polak, J. M. Cloning and tissue distribution of three murine alpha/beta hydrolase fold protein cDNAs. *Biochem Biophys Res Commun* **292**, 617-25 (2002).
36. Zhang, L., Godzik, A., Skolnick, J. & Fetrow, J. S. Functional analysis of the Escherichia coli genome for members of the alpha/beta hydrolase family. *Fold Des* **3**, 535-48 (1998).
37. Huttner, K. M. & Ouellette, A. J. A family of defensin-like genes codes for diverse cysteine-rich peptides in mouse Paneth cells. *Genomics* **24**, 99-109 (1994).
38. White, S. H., Wimley, W. C. & Selsted, M. E. Structure, function, and membrane integration of defensins. *Curr Opin Struct Biol* **5**, 521-7 (1995).
39. Hornef, M. W., Putsep, K., Karlsson, J., Refai, E. & Andersson, M. Increased diversity of intestinal antimicrobial peptides by covalent dimer formation. *Nat Immunol* **5**, 836-43 (2004).
40. Schable, K. F. et al. Characteristics of the immunoglobulin Vkappa genes, pseudogenes, relics and orphons in the mouse genome. *Eur J Immunol* **29**, 2082-6 (1999).
41. Rankin, T. L., Ong, D. E. & Orgebin-Crist, M. C. The 18-kDa mouse epididymal protein (MEP 10) binds retinoic acid. *Biol Reprod* **46**, 767-71 (1992).
42. Rankin, T. L., Tsuruta, K. J., Holland, M. K., Griswold, M. D. & Orgebin-Crist, M. C. Isolation, immunolocalization, and sperm-association of three proteins of 18, 25, and 29 kilodaltons secreted by the mouse epididymis. *Biol Reprod* **46**, 747-66 (1992).
43. Brooks, D. E. The major androgen-regulated secretory proteins of the rat epididymis bear sequence homology with members of the alpha 2u-globulin superfamily. *Biochem Int* **14**, 235-40 (1987).
44. Lareyre, J. J. et al. Molecular cloning and hormonal regulation of a murine epididymal retinoic acid-binding protein messenger ribonucleic acid. *Endocrinology* **139**, 2971-81 (1998).
45. Zwain, I. H., Grima, J. & Cheng, C. Y. Rat epididymal retinoic acid-binding protein: development of a radioimmunoassay, its tissue distribution, and its changes in selected androgen-dependent organs after orchiectomy. *Endocrinology* **131**, 1511-26 (1992).
46. Eguchi, Y. et al. Expression of lipocalin-type prostaglandin D synthase (beta-trace) in human heart and its accumulation in the coronary circulation of angina patients. *Proc Natl Acad Sci U S A* **94**, 14689-94 (1997).
47. Tanaka, T. et al. Lipocalin-type prostaglandin D synthase (beta-trace) is a newly recognized type of retinoid transporter. *J Biol Chem* **272**, 15789-95 (1997).
48. Cho, Y., Batt, C. A. & Sawyer, L. Probing the retinol-binding site of bovine beta-lactoglobulin. *J Biol Chem* **269**, 11102-7 (1994).
49. Kastner, P. et al. Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003 (1994).
50. Gruber, P. J. et al. RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J Clin Invest* **98**, 1332-43 (1996).
51. Brown, L., Espinosa, R., 3rd, Le Beau, M. M., Siciliano, M. J. & Baer, R. HEN1 and HEN2: a subgroup of basic helix-loop-helix genes that are coexpressed in a human neuroblastoma. *Proc Natl Acad Sci U S A* **89**, 8492-6 (1992).
52. Lipkowitz, S. et al. A comparative structural characterization of the human NSCL-1 and NSCL-2 genes. Two basic helix-loop-helix genes expressed in the developing nervous system. *J Biol Chem* **267**, 21065-71

(1992).
53. Jing, E., Nillni, E. A., Sanchez, V. C., Stuart, R. C. & Good, D. J. Deletion of the Nhlh2 transcription factor decreases the levels of the anorexigenic peptides alpha melanocyte-stimulating hormone and thyrotropin-releasing hormone and implicates prohormone convertases I and II in obesity. *Endocrinology* **145**, 1503-13 (2004).
54. Coyle, C. A. et al. Reduced voluntary activity precedes adult-onset obesity in Nhlh2 knockout mice. *Physiol Behav* **77**, 387-402 (2002).
55. Alliel, P. M., Perin, J. P., Jolles, P. & Bonnet, F. J. Testican, a multidomain testicular proteoglycan resembling modulators of cell social behaviour. *Eur J Biochem* **214**, 347-50 (1993).
56. Charbonnier, F. et al. Expression of the proteoglycan SPOCK during mouse embryo development. *Mech Dev* **90**, 317-21 (2000).
57. Bonnet, F. et al. Structure and cellular distribution of mouse brain testican. Association with the postsynaptic area of hippocampus pyramidal cells. *J Biol Chem* **271**, 4373-80 (1996).
58. Charbonnier, F., Perin, J. P., Roussel, G., Nussbaum, J. L. & Alliel, P. M. [Cloning of testican/SPOCK in man and mouse. Neuromuscular expression perspectives in pathology]. *C R Seances Soc Biol Fil* **191**, 127-33 (1997).
59. Cifuentes-Diaz, C. et al. Regulated expression of the proteoglycan SPOCK in the neuromuscular system. *Mech Dev* **94**, 277-82 (2000).
60. Nash, S. R. et al. Cloning, gene structure and genomic localization of an orphan transporter from mouse kidney with six alternatively-spliced isoforms. *Receptors Channels* **6**, 113-28 (1998).
61. Kiss, H. et al. The LZTFL1 gene is a part of a transcriptional map covering 250 kb within the common eliminated region 1 (C3CER1) in 3p21.3. *Genomics* **73**, 10-9 (2001).
62. Shioda, T. et al. Synthesis and accumulation of thiamin triphosphate in Escherichia coli cells expressing chicken cytosolic adenylate kinase. *Biochim Biophys Acta* **1115**, 36-41 (1991).
63. Bettendorff, L. Thiamine homeostasis in neuroblastoma cells. *Neurochem Int* **26**, 295-302 (1995).
64. Fankhauser, H., Zurlinden, A., Schweingruber, A. M., Edenharter, E. & Schweingruber, M. E. Schizosaccharomyces pombe thiamine pyrophosphokinase is encoded by gene tnr3 and is a regulator of thiamine metabolism, phosphate metabolism, mating, and growth. *J Biol Chem* **270**, 28457-62 (1995).
65. Holzer, H. & Gubler, C. Thiamin pyrophosphate: catalytic mechanism, role in protein turnover. *J Nutr Sci Vitaminol (Tokyo)* **Spec No**, 287-91 (1992).
66. Hohmann, S. & Meacock, P. A. Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast Saccharomyces cerevisiae: genetic regulation. *Biochim Biophys Acta* **1385**, 201-19 (1998).
67. Fawns, H. Vitamin B1 and beriberi. *Nurs Times* **71**, 1870-3 (1975).
68. Traviesa, D. C. Magnesium deficiency: a possible cause of thiamine refractoriness in Wernicke-Korsakoff encephalopathy. *J Neurol Neurosurg Psychiatry* **37**, 959-62 (1974).
69. Nosaka, K. et al. Molecular cloning and expression of a mouse thiamin pyrophosphokinase cDNA. *J Biol Chem* **274**, 34129-33 (1999).
70. Farlie, P. et al. Ypel1: a novel nuclear protein that induces an epithelial-like morphology in fibroblasts. *Genes Cells* **6**, 619-29 (2001).
71. Carninci, P. & Hayashizaki, Y. High-efficiency full-length cDNA cloning. *Methods Enzymol* **303**, 19-44 (1999).
72. Gould, A. P., Brookman, J. J., Strutt, D. I. & White, R. A. Targets of homeotic gene control in Drosophila. *Nature* **348**, 308-12 (1990).
73. Huang, C. et al. Embryonic atrial function is essential for mouse embryogenesis, cardiac morphogenesis and angiogenesis. *Development* **130**, 6111-9 (2003).
74. Fishman, M. C. & Chien, K. R. Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-117 (1997).

CHAPTER 5

Chapter 5

Sexual behaviour and odour preference in *Sp4* knockout mice

Pieter Fokko van Loo,[1] Marja Ooms,[2] Jan Vreeburg,[2] Sjaak Philipsen,[1] and Julie Bakker[3]

[1] Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands
[2] Department of Reproduction and Development, Erasmus MC, Rotterdam, The Netherlands
[3] Centre for Cellular and Molecular Neurobiology, University of Liege, Liege, Belgium

Abstract

Mice lacking the Krüppel-like zinc finger transcription factor specificity protein 4 (Sp4) do not produce offspring. The Sp4 protein has a versatile expression pattern that includes male and female gonads and central nervous system. The infertility of the *Sp4*$^{-/-}$ mice in combination with the expression pattern of Sp4 prompted us to investigate their mating behaviour, and their sociosexual behaviour at adulthood. We made use of a complete *Sp4* knockout mouse line in the fvb/n genetic background, and analysed the mating behaviour of gonadally intact *Sp4*$^{-/-}$ males, and ovariectomized *Sp4*$^{-/-}$ females after treatment with estradiol and progesterone. We found an almost complete absence of mounting behaviour of the Sp4-*null* males, and a dramatic reduction in lordosis behaviour of the *Sp4* knockout females. Additional experiments revealed that the male *Sp4* knockout mice displayed a lack of interest to investigate volatile and non-volatile odours derived from conspecifics. However, female Sp4-*null* mice did not differ from wild-type mice in olfactory investigation. Thus, in contrast to female *Sp4* knockout mice, the absence of sexual behaviour of male *Sp4* knockout mice might partly be due to a deficit in the olfactory system.

At the end of the experiment, the testes and seminal vesicles of the 7-month-old *Sp4*$^{-/-}$ males appeared significantly smaller than those of their wild-type littermates. In contrast, their body weights were higher, probably due to an increase in white adipose tissue. Daily administration of estradiol for 7 days to the ovariectomized females demonstrated that uteri of *Sp4*$^{-/-}$ mice have a reduced sensitivity to estrogen, suggesting that the mechanism of action of estradiol is disturbed in *Sp4* knockout mice.

Introduction

Transcription factor specificity protein 4 (Sp4) belongs to the Sp/XKLF (specificity protein/ x Krüppel-like factor) family. The family consists of 25 factors, sharing a conserved C-terminally located DNA-binding domain.[1] This domain is composed of three Cys_2-Hys_2 zinc-fingers, similar to those found in the *Drosophila* Krüppel segmentation protein.[2] The DNA-binding domain recognizes the GC- (GGGGCGGGG) and GT- (GGTGTGGGG) boxes,[3-6] which are abundantly present in the regulatory elements of vertebrate genomes.[7]

The Sp subclass of the Sp/XKLF family consists of nine Sp factors (Sp1-9).[8] Sp1 and Sp3 have a structural composition similar to Sp4, comprising two N-terminally located glutamine-rich activation domains and the zinc-finger region.[7] However, each factor has a distinct physiological function, and Sp4 has a different expression pattern from Sp1 and Sp3. Whereas Sp1 and Sp3 are ubiquitously expressed, Sp4 displays a dynamic expression pattern that starts in the posterior neuropore, in the mouse at embryonic day 9 (E9), followed by expression in the developing brain, neural tube and central nervous system during the progression of embryogenesis. Additional embryonic expressing sites are tooth buds, testes, ovaries, nasal mucosa, vomeronasal organ (VNO), and cardiac conduction system. At adulthood, Sp4 is most prominently expressed in brain, skeletal muscle and epithelial tissues.[9-12]

The phenotype of *Sp4*$^{-/-}$ mice is very different from the phenotypes of *Sp1* and Sp3-*null* mice. *Sp1*$^{-/-}$ embryos are severely growth retarded, and die around E10. *Sp3*$^{-/-}$ embryos are mildly growth retarded, display multiple developmental defects including disturbed tooth- and lung development, and impaired bone ossification and hematopoiesis.[13-15] Sp3-*null* mutants die perinatally[14] of severe cardiac malformations (van Loo et al., this thesis). In contrast, *Sp4*$^{-/-}$ mice are born at a normal Mendelian ratio. The data on the phenotypes of *Sp4* mutant mice are rather confusing, as three different mouse lines of Sp4 have been generated in three different genetic backgrounds. One *Sp4* mutant line lacks the DNA binding domain.[12] The other two *Sp4* mutant lines are complete *Sp4* knockout lines.[10,11] Supp and Göllner reported that 2/3 of the *Sp4*-deficient pups die within the first weeks after birth,[10,12] in contrast to Nguyen-Tran and coworkers, who reported an increased mortality between 6-8 months of age. They revealed that this is caused by cardiac arrhythmia, attributed to a defect in the cardiac conductive system.[11] The surviving *Sp4*-deficient pups have a transient growth arrest, but they never catch up completely with wild-type littermates.[10] The diversity in the descriptions of the phenotype of *Sp4*-deficient mice may be explained partially by the different genetic backgrounds in which the *Sp4* mutant allele is present (129/Ola with CF-1 for Supp, 129/Ola with C57Bl/6 for Göllner and J1 129SVJ crossed with Black Swiss for Nguyen-Tran). In addition, each laboratory has performed different analyses, thus revealing specific aspects of the phenotype.

A striking aspect of the Sp4-*null* phenotype is the infertility of the Sp4-*null* males;[10,12] they consistently fail to produce offspring. Probably, the *null* males would fertilize females if they could produce copulation plugs, since their testes showed a normal spermatogenesis

and their reproductive tracts appear to be normal. Supp et al. (1996) found an absence of mounting behaviour of the *Sp4*$^{-/-}$ males following the introduction of superovulated wild-type female mice into the cage of the mutant males,[12] suggesting that their infertility might be caused by a failure to copulate. Data about the mating behaviour of Sp4-*null* males of the other mouse lines are not available. Depending on the *Sp4* mutant line used, the *Sp4*-deficient females were reported to display normal fertility,[12] or had only sporadically a litter.[10] The latter is probably related to their pronounced delayed vaginal opening time and small uterus size. Data concerning mating behaviour of the *Sp4*$^{-/-}$ females are currently not available.

To further elucidate the biological function of Sp4, we have characterized the disturbed reproduction of the *Sp4*-deficient mice, with the complete *Sp4* knockout mouse line of Göllner et al. (2001) crossed back in the fvb/n genetic background. We analysed mating behaviour of gonadally intact males, since our data (not published) revealed no or only a slight reduction of the weights of the testes and seminal vesicles of maturating *Sp4*$^{-/-}$ males, indicating a normal hypothalamo-pituitary-gonadal axis. In contrast, the *Sp4*$^{-/-}$ females were ovariectomized and treated with estradiol and progesterone to induce estrous behaviour. The outcomes of the study of sexual behaviour prompted us to investigate the odour perception of *Sp4*-deficient males and females.

Results

Male and female sexual behaviour

The previous analysis of the *Sp4* knockout mouse showed impaired sexual development and fertility in females,[10] and a consistent failure of the males to produce offspring.[12] We therefore asked if the lack of reproduction of the Sp4-*null* mice could have its origin in aberrant sexual behaviour of these animals. To answer this question, we examined the coital behaviours and the lordosis frequency of *Sp4* knockout males and females, respectively, by behaviour experiments with gonadally intact *Sp4*$^{-/-}$ male and ovariectomized *Sp4*$^{-/-}$ female treated with high levels of estrogen.

Male coital behaviour. Male coital behaviour, such as mounts and intromissions, were observed in all wild-type males (Figure 1a,b). By contrast, only one *Sp4*$^{-/-}$ mouse showed some male coital behaviour albeit not during all 5 tests: in test 2 and 3, the animal mounted the estrous female only once, whereas in test 4 the animal mounted the female a total of 17 times as well as intromitted her a total of 7 times. However, this animal never copulated until ejaculation. By contrast, 6 out of 9 wild-type males ejaculated at least once. Two-way ANOVA (analysis of variance) with genotype as independent factor and test as repeated factor on the number of mounts showed a significant genotype effect ($F(1,51)= 21.84$, $p=0.0002$), a significant effect of repeated testing ($F(3,51)= 7.27$, $p=0.0004$) and a significant genotype X test interaction ($F(3,51)= 6.64$, $p=0.007$). Post hoc analysis indicated that Sp4-*null* mice displayed significantly fewer mounts than wild-type mice and that the number of mounts

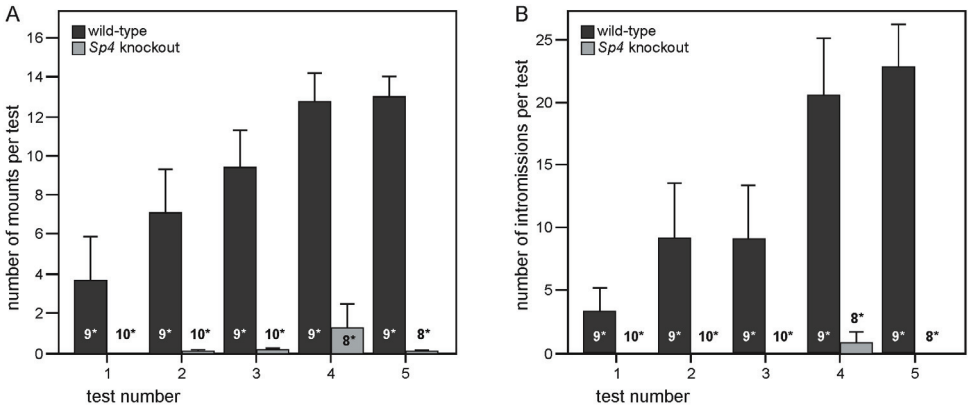


Figure 1. Male coital behaviour
Gonadally intact wild-type and *Sp4* knockout males were tested for male coital behaviour with an estrous female in five sequential tests. Given are the mean number of mounts (a) and intromissions (b) per genotype and test number. Asterisks indicate numbers of mice per group; error bars are standard error of the mean.

increased significantly over tests in wild-type but not Sp4-*null* mice. Two-way ANOVA on the number of intromissions revealed a significant effect of genotype ($F(1,51)=60.83$, $p=0.0001$), a significant effect of repeated testing ($F(3,51)=6.0$, $p=0.0014$) and a significant genotype X test interaction ($F(3,51)=4.57$, $p=0.0065$). Post-hoc analysis showed that Sp4-*null* mice displayed fewer intromission that wild-type males and that the number of intromissions increased over testing wild-type mice but not in tests with Sp4-*null* mice.

Female coital behaviour. The numbers of lordosis responses in 3 consecutive tests were pooled. By doing so, all females were mounted at least 10 times. The wild-type females received on average 20.6 ± 2.2 mounts whereas $Sp4^{-/-}$ mice received on average 18.2 ± 1.7 mounts (no statistically significant differences in number of mounts received between wild-type and *Sp4* knockout females). As shown in Figure 2, lordosis quotients as calculated by the number of lordosis divided by the number of mounts x 100% were much lower in Sp4-*null* females compared to wild-type females. One-way ANOVA showed a significant effect of genotype ($F(1,16)=92.1$, $p=0.0001$).

In summary, these experiments show that reduced reproduction of the *Sp4* knockout mice is related to impaired sexual behaviour of both sexes, as the Sp4-*null* males lack mounting behaviour, and the $Sp4^{-/-}$ females display a significant reduction in lordosis behaviour.

Odour preferences of *Sp4* knockout mice

The olfactory system of many mammalian species facilitates several aspects of social behaviour, including the determination of sexual partner preference.[17,18] The preference for a partner is determined by volatile- and non-volatile odours that are scattered by conspecifics. In rodents, the majority of these olfactory signals are detected and processed by a polysynaptic accessory olfactory system (AOS).[19,20] As our results show that the $Sp4^{-/-}$ mice display aberrant

sexual behaviour, we assessed if this defect may have its origin in impaired olfactory communication. We therefore examined the preference of the *Sp4* knockout animals for volatile and non-volatile odours of an intact male or estrous female mouse.

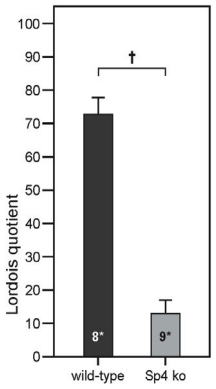


Figure 2. Female coital behaviour
The lordosis quotients for wild-type and *Sp4*$^{-/-}$ females, calculated as the number of lordosis divided by the number of mounts received. The lordosis quotients were calculated from the pooled data of three successive tests. Asterisks indicate numbers of mice per group; error bars are standard error of the mean; † Significant ($P < 0.05$) difference between genotype; Statistical analysis by ANOVA.

Odour preferences of males. Male wild-type and *Sp4* knockout mice were tested for their preference for volatile body odours derived from either an intact male or estrous female mouse. ANOVA analysis on the time the males spent sniffing these odours showed a significant effect of genotype ($F(1,16)=60.5$, $p=0.0001$), but no significant effect of kind of odour (male of female) stimulus nor an interaction. Posthoc analysis revealed that Sp4-*null* males spent much less time investigating volatile odours than wild-type males did. However, none of the genotypes showed a strong preference for either odour (Figure 3a). When subjects were tested for their preference for soiled bedding (containing predominantly non-volatile odours) derived from either intact male or estrous female mice, it was again found that Sp4-*null* males spent much less time investigating both odour stimuli than wild-type subjects did (Figure 3b). In contrast to volatile odours, wild-type but not *Sp4*$^{-/-}$ males showed a strong preference to investigate soiled bedding derived from estrous females over that from intact males. Accordingly, ANOVA showed a significant effect of genotype ($F(1,15)= 92.4$, $p=0.0001$), a significant effect of odour stimulus ($F(1,15)=28.3$, $p=0.0001$) and a significant genotype x odour stimulus interaction ($F(1,15)=20.2$, $p=0.0004$).

Odour preference of females. In contrast to males, Sp4-*null* females spent equal time investigating the volatile odours of male or female mice as wild-type females did. ANOVA

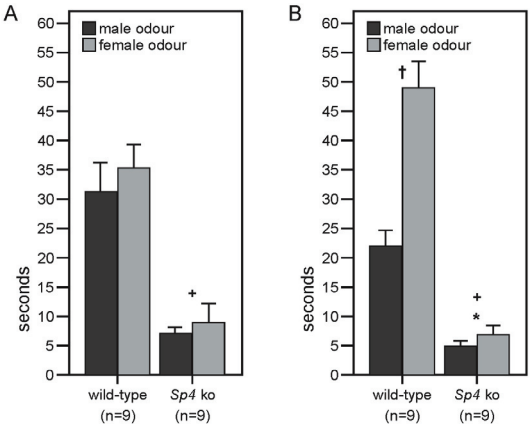


Figure 3. Odour preference of males
The mean amount of time in seconds that gonadally intact wild-type and *Sp4* knockout males spent investigating volatile olfactory cues (a) and non-volatile olfactory cues (b), when given a choice between odours from either an anesthetized intact male or from an anesthetized estrous female in a Y-maze set-up during a 5 minute test. Error bars are standard error of the mean. n: number of animals investigated in each group; + Significantly ($P < 0.05$) different from wild-type males in time investing odours; † Significantly ($P < 0.05$) different preference for male or female odour; * Significantly ($P < 0.05$) different form wild-type males, same odour stimulus; statistical analysis by ANOVA.

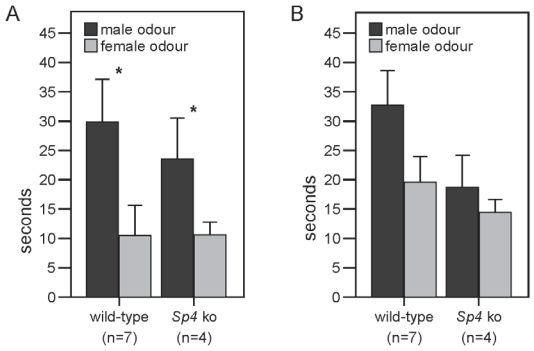


Figure 4. Odour preference of females
The mean amount of time in seconds that ovariectomized wild-type and *Sp4* knockout females spent investigating volatile olfactory cues (a) and non-volatile olfactory cues (b), when given a choice between odours from either an anesthesized intact male or from an anesthetized estrous female in a Y-maze set-up during a 5 minute test. Females were implanted with a Silastic capsule containing 17β-estradiol and 2-4 hours prior testing injected with 500 µg progesterone. Error bars are standard error of the mean. n: number of animals investigated in each group; * Significantly ($P < 0.05$) different preference for male or female odour; statistical analysis by ANOVA.

only showed a significant effect of odour stimulus ($F(1,9)=6.2$, $p=0.03$). All females showed a preference to investigate volatile body odours from an intact male over those from an estrous female (Figure 4a). When females were offered a choice between soiled bedding from males and females, again Sp4-*null* females spent similar amounts of time investigating the bedding as the wild-type females did. ANOVA showed no significant effect of genotype or odour stimulus or an interaction between the two factors. There was a slight preference, albeit not significant, to investigate male-soiled bedding (Figure 4b).

In summary, whereas the *Sp4*$^{-/-}$ males display in general a diminished interest in investigating any kind of the odours offered, the *Sp4*$^{-/-}$ females do not display a significant difference in their behaviour to volatile and non-volatile odours of either intact males or estrous females. Importantly, the *Sp4* knockout males display an impaired preference to non-volatile odours that normally induce coital behaviour by male mice, since they have a no preference for an estrous female over an intact male.

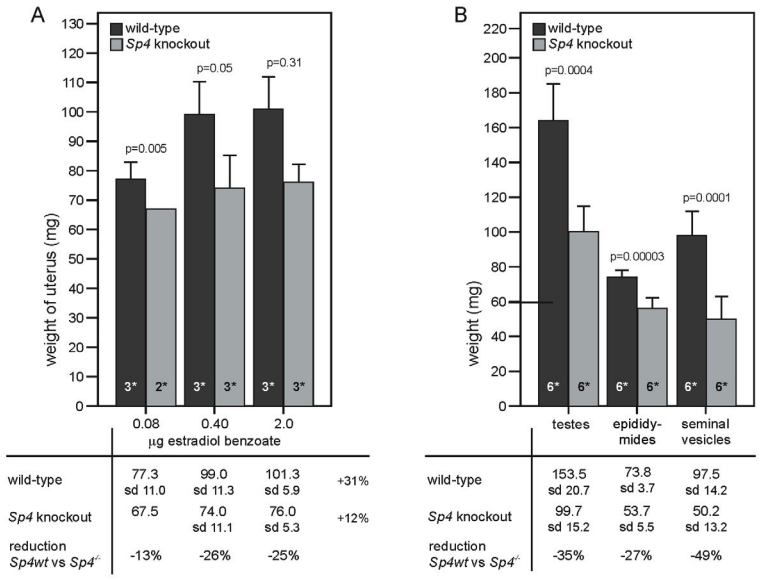
Gonads and steroid hormones

Stimulation of the uterus with estrogens. Studies with knockout mice have revealed that estrogens influence numerous target tissues in the reproductive systems of females and male, such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate.[21-26] In the uterus, estrogens induce a growth response along with the onset of expression various genes, including the genes encoding the progesterone receptor.[27] Since the uterus of *Sp4* knockout female has an immature appearance, we assessed if this immature status is an effect of aberrant function of the cascade that is induced by estrogens. Therefore, ovariectomized wild-type and *Sp4*-deficient females of 24 weeks old were treated daily with 0.08, 0.4, or 2.0 µg estradiol benzoate over a period of 1 week, and subsequently killed and examined. The wild-type control females experienced the expected dosage-dependent growth of their uteri after the administration of estradiol benzoate over a period of seven days (Figure 5a). The uteri of the wild-type mice treated with 0.08, 0.4, and 2.0 µg estradiol benzoate weighted 77.3 (sd 11.0), 99.0 (sd 11.3), and 101.3 (sd 5.9) mg, respectively. Thus, administration of

0.4 or 2.0 µg of estradiol benzoate results in an increase in uterus weight of 28% or 31%, respectively, compared to administration of 0.08 µg of estradiol benzoate.

The *Sp4* knockout females displayed a blunted dosage-dependent increase of uterus weight (Figure 5a), in particular when the same doses of estradiol benzoate are compared (13% with p=0.31 for 0.08 µg, 26% with p=0.05 for 0.4 µg, 25% with p=0.005 for 2.0 µg). This is despite the fact that their bodyweights are only slightly reduced in comparison to the wild-type females from the same litters (from 27.4 to 26.3 g with p=0.049) (Figure 6). Even the high quantity of 2.0 µg estradiol benzoate (a quantity exceeding normal physiological amounts) is unable to provoke an *Sp4*-deficient uterus to gain a similar amount of weight as observed in the wild-type situation. Taken together, these results show that the uteri of the Sp4-*null* females display a significantly reduced sensitivity to estrogens.

Testosterone, which is generally thought to be necessary for male sexual behaviour at adulthood,[28-30] is produced in the testis by the interstitial Leydig cells. We therefore examined the testis as well as the closely related genital organs of 7-months-old *Sp4*$^{-/-}$ males. The testis *Sp4*$^{-/-}$ males weighed on average 99.7 mg (sd 13.2). This is a weight reduction of more than 35% (p=0.004), as the testis of wild-type males weighed on average 153.5 mg (sd 20.7) (Figure 5b). The epididymides and seminal vesicles of the *Sp4*$^{-/-}$ males are also reduced in weight, with, respectively, an average reduction of 27% (p=0.0003) and 49% p=0.0001) percent compared to wild-type littermates (Figure 5b). The epididymides of a *Sp4* knockout males



	0.08 µg	0.40 µg	2.0 µg	
wild-type	77.3 sd 11.0	99.0 sd 11.3	101.3 sd 5.9	+31%
Sp4 knockout	67.5	74.0 sd 11.1	76.0 sd 5.3	+12%
reduction *Sp4wt* vs *Sp4*$^{-/-}$	-13%	-26%	-25%	

	testes	epididymides	seminal vesicles
wild-type	153.5 sd 20.7	73.8 sd 3.7	97.5 sd 14.2
Sp4 knockout	99.7 sd 15.2	53.7 sd 5.5	50.2 sd 13.2
reduction *Sp4wt* vs *Sp4*$^{-/-}$	-35%	-27%	-49%

Figure 5. Comparison of genital organs
(a) The average weight of wild-type and *Sp4* knockout uteri after 7 days treatment with different quantities of estradiol benzoate. (b) The mean weight of two testes, two epididymides and two seminal vesicles of 30 weeks old wild-type and *Sp4* knockout males. P-values indicate to what extent the weight of the tissues of the wild-type and *Sp4*$^{-/-}$ mice significantly differ from each other, per condition. Asterisks indicate numbers of mice per group; sd: standard deviation; error bars are standard deviation; statistics by Student's t-test.

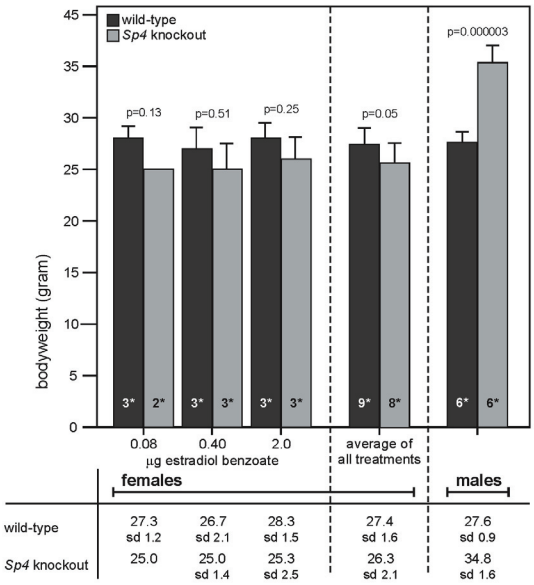


Figure 6. Body weight at late adulthood
The mean body weight of 24 weeks old female and 30 weeks old male wild-type and *Sp4*$^{-/-}$ mice. P-value indicates to what extent the body weights of the wild-type and *Sp4*$^{-/-}$ mice significantly differ from each other, per given condition. Asterisks indicate numbers of mice per group; sd: standard deviation; error bars are standard deviation; statistics by Student's t-test.

weighed on average 53.7 mg (sd 5.5) versus 73.8 mg (sd 14.2) for wild-type epididymides, whereas the seminal vesicles of the *Sp4*$^{-/-}$ weighed on average 50.2 mg (sd 13.2) versus the averaged weight of 97.5 mg (sd 14.2) of the seminal vesicles of wild-type males (Figure 5b). These reductions in tissue size are in sharp contrast to the increased bodyweight of the *Sp4* knockout males. They have, when they are seven months old, an average bodyweight of 34.9 gram (sd 1.6). This is an increase of 25% (p=0.00003) compared to wild-type littermates, who have an average bodyweight of 27.6 gram (sd 0.9). As we observed an abundant amount of white adipose tissue in these *Sp4*$^{-/-}$ males at the time of dissection, the increased bodyweight might be the result of the accumulation of white adipose tissue.

Discussion

Absence of coital behaviour

The present study shows that the attempts to breed with adult *Sp4*$^{-/-}$ males have been unsuccessful owing to the absence of coital behaviour. This behavioural deficit could be the consequence of a several of causes. One of them is a severely impaired ability to smell volatile and non-volatile odours of conspecifics or the absence of motivation to investigate these odours. Mice have a dual olfactory system, consisting of the main olfactory system, which is capable of responding to volatile odours and the accessory olfactory system, which is thought to respond to volatile and non-volatile odours.[31] The *Sp4* knockout males spend appreciably less time sniffing volatile and non-volatile odours of gonadally intact males or estrous females than their wild-type littermates. In addition, they showed no preference for non-volatile odours of estrous females over non-volatile odours of gonadally intact males,

while wild-type males do display such a preference. Thus, our data indicate that Sp4-*null* males have a dysfunctional main- and accessory olfactory system. The specific role of the main olfactory system and the accessory olfactory system for male mating behaviour is not fully clear. In the male hamster, lesions of both the main- as well as the accessory olfactory system are needed to eliminate consistently mating behaviour.[32] A strong reduction in male mating behaviour has also been found in the rat after bulbectomy.[31] Recently, it was found that mating behaviour of male mice was not affected by surgical removal of the vomeronasal organ.[33] This corroborated earlier findings in male Trp2-*null* (transient receptor potential 2) mice.[34,35] These mice are characterized by a non-functional vomeronasal organ, but they display normal levels of male mating behaviour. Together, these observations suggest that in mice elimination of male sexual activity results from non-function of the main olfactory system as well as the vomeronasal system . Besides the deficits in the olfactory system, other factors might equally be responsible for the absence of sexual behaviour since *Sp4*-deficient female mice also display severely impaired lordosis behaviour. However, their aberrant sexual behaviour does not appear to result from defects in the perception of socially relevant odours, since their response to sexually-related volatile- and non-volatile odours is not significant different from wild-type females.

The absence of both male and female sexual behaviour in mice as a consequence of a non-endocrine manipulation indicates that a fundamental process in the brain, needed for the expression of male and female sexual behaviour, might be disturbed in the *Sp4* knockout. For this behaviour it is of vital importance that brain structures that mediate in this behaviour in adulthood develop correctly during brain development early in life. Recently, it has been found, that estradiol is an important mediator in this process, by studies on knockout females of the *aromatase* (C*yp19*) gene.[36,37] These females, which lack the enzyme that aromatizes testosterone into estradiol,[38,39] display at adulthood a reduced female and male sexual behaviour after treatment with estradiol and progesterone, and estradiol and testosterone, respectively.[40] Whereas wild-type females display male and female sexual behaviour after appropriate hormonal stimulation. These observations indicate that during embryonic life estradiol is needed for the correct development of the mouse brain as a sexual organ. Since Sp4 is expressed in the brain of mouse embryos[10,12] and Sp1 like factors might be involved in the mechanism of action of estradiol,[41] it is possible that the deficiency of Sp4 affect the normal brain development and thereby bring about the absence of normal sexual behaviour of these animals. To address this topic, first the exact expression pattern of the Sp4 protein in the brain in the sequential stages of life should be elucidated. Namely, today it is only known that Sp4 is expressed in brain during development and at adulthood,[10,12] but a detailed expression pattern is unknown.

Sex hormones are not solely important in the developmental process of the brain, but also in the execution of the physiological processes in the brain that lie behind sexual behaviour.[37,42-44] The impaired coital behaviour of the Sp4-*null* line may have its origin at this level. Such information comes from the *aromatase* knockout males. These males lack capacity

to aromatize testosterone into estradiol in the brain. This defect causes a similar phenotype as we have found in the *Sp4* knockout males: impaired discrimination between conspecifics through olfactory and visual cues, and the lack of normal coital behaviour.[36,37]

Role of ER signalling

More data support the hypothesis that the aberrant reproduction of *Sp4* knockout line is related to sex hormones. Namely, desides the fact that uteri of *Sp4*$^{-/-}$ females have an immature appearance at around 8 weeks of age,[10] we show here that their uteri are blocked in execution of the developmental program that is normally induced by estrogens. This block is not complete, because estrogen-treated *Sp4*$^{-/-}$ uteri still gain weight in a dose- dependent fashion (Figure 5). Thus, Sp4 deficiency affects development of the uterus, resulting in an apparently largely dysfunctional uterus in the end.

This anomaly could either be brought about by a defect in estrogen signalling or by a defect in a downstream action that is normally induced by estrogen. Abnormality in the estrogen cascade is a plausible hypothesis, since the analysis of *ERα*$^{-/-}$ and *ERβ*$^{-/-}$ mice has shown that the lack of proper estrogen signalling in the uterus affects maturation, resulting in an immature uterus.[22,23] Thus, expression of the ER itself might be affected by Sp4 deficiency, since Sp4 is expressed in the uterus.[12] Gel mobility shift assays, transactivation studies and pull-down experiments on material obtained from cultured cells do indeed suggest that Sp factors, mainly Sp1 and Sp3, are involved in regulation of ER expression.[41,45-47] The composition of Sp4 is highly similar to the ubiquitously expressed Sp1 and Sp3 proteins.[48-50] It is therefore a generally accepted hypothesis that genes harbouring GC- and GT-boxes in their regulatory elements are not targeted by a single Sp factor, but by a number of Sp factors. In view of this, it is likely that, besides Sp1 and Sp3, Sp4 might be involved in the expression of the ERs.

Alternatively, it is possible that distribution of the ER is not affected in the *Sp4*$^{-/-}$ mice, but that the expression of target genes of the ER signalling pathway are affected. Functional interactions between the ER and either Sp1 or Sp3 in the regulation of target genes of the ER-cascade have been described by a number of laboratories (e.g. references [51-57]). It is therefore possible that Sp4, which we have shown to be important for sexual maturation and development of sexual behaviour, is involved in activation of target genes of the ER pathway. It is not known if transcription factors Sp1 and Sp3 are involved in reproduction, since the premature death of the *Sp1* and *Sp3* mouse knockout lines excludes an assessment of reproductive fitness. The *Sp1* knockout mouse is already severely retarded during early embryonic development and dies around E10.[58] The *Sp3*$^{-/-}$ mouse, however, survives until late embryogenesis, but succumbs to perinatal lethality due to severe dysfunction of the heart (Chapter 3, this thesis). However, although the knockout mouse lines from these two proteins do not give a clue if they are functional in tissues of the reproductive system, the ubiquitous expression pattern of these two proteins suggests that they might be deployed in these tissues, in addition to Sp4.

Late-onset obesity

The late-onset of obesity by the *Sp4* knockout males during late adulthood is remarkable, since the *Sp4*$^{-/-}$ animals display a growth arrest during their early life-phase.[10] Late-onset obesity in males has also been reported in mouse models with aberrant sex hormone signalling. The *ERα*$^{-/-}$ mouse develops progressive obesity with advancing age, starting after the stage of sexual maturation. This obesity is a result of the growth of the white adipose tissue, but not of the brown adipose tissue.[59-61] Furthermore, Sato et al. (2003) showed that deficiency of the *androgen receptor* (*AR*) also gives rise to obesity during adulthood, but solely by males. This obesity is also, like in the *ERα*$^{-/-}$ mice, a result of accumulation of white adipose tissue.[62] In the *Sp4*-deficent males, the obesity with late-onset may also be the result of an increase of white adipose tissue, as we have observed a growth of their white adipose tissue. This suggests, in combination with the infertility of the *Sp4*-deficienct males, that a sex steroid hormones cascade is aberrant in the *Sp4* knockout mouse, similar to the *AR* or *ERα* knockout mouse lines. Possibly, the cause of the obesity for the *Sp4* knockout males is related to the reduced testis size, since this tissue is the main source of the androgenic hormone testosterone. However, it must be noted that the testes of *Sp4*-deficient males have a normal morphology and produce motile sperm. In conclusion, the observed late-onset obesity in males is in agreement with the hypothesis that transcription factor Sp4 contributes to functional signalling of sex steroid hormone pathways.

In summary, we have shown that the severely impaired reproduction capacity of the *Sp4* knockout mouse line is brought about by dysfunction of a number of physiological processes. The data support the hypothesis that these abnormalities might be brought about by dysfunction of the signalling cascades that rely on activation by steroid sex hormones.

Materials and Methods

Animals

Sp4 knockout mice[10] were back-crossed for more than 10 generations to fvb/n mice. Heterozygous *Sp4* males and females were bred to generate wild-type, heterozygous and Sp4- *null* offspring. Genotyping was performed by polymerase chain reaction (PCR) analysis of tail DNA, using 3 different primers: a sense primer in the *Sp4* gene amplifying the wild-type allele (5'-CAAACTGTCCAGGTCCAGAA-3'), a sense primer in the *Neo* gene amplifying the knockout allele (5'-AGCGCATCGCCTTCTATC-3'), and a common antisense primer in the *Sp4* gene (5'-CTCACAACCATATACCAATGCAAG-3'). PCR conditions: 94°C for 45 seconds, 54°C for 45 seconds and 72°C for 30 seconds for 34 cycles. All experimental males were housed alone whereas the females were housed in groups of 3-4 (genotypes mixed). All animals were kept under a reversed light-dark cycle (12:12 LD). Food (regular mouse chow) and water were always available ad libidum.

Stimulus males and females were derived from a C57Bl/6 breeding colony at the Center for Cellular and Molecular Neurobiology, University of Liege, Belgium. The stimulus females were ovariectomized in adulthood under general anesthesia using a mixture of ketamine

(200 µg/g, per mouse, intraperitoneally, i.p.) and medetomidine (Domitor® from Pfizer, 8.3 µg/g, per mouse, i.p.). Mice received atipamezole (antisedan® from Pfizer, 2.5 µg/g per mouse, subcutaneously, s.c.) at the end of the surgery to antagonize medetomidine-induced effects, thereby accelerating their recovery. At the time of ovariectomy, they were implanted subcutaneously with a Silastic capsule (inner diameter: 1.57 mm; outer diameter: 2.41 mm; length: 5 mm) containing crystalline 17β-estradiol diluted 1:1 with cholesterol). Three hours before testing, stimulus females were injected sc with 500 µg progesterone. Stimulus males were left gonadally intact.

All behavioural tests were conducted between 13:00 and 18:00h during the dark phase of the LD cycle. Furthermore, no behavioural testing was conducted during the 24h following cleaning of the subjects' cages.

Analysis of coital behaviour of male and female Sp4-*null* mice

Male coital behaviour. Gonadally intact male wild-type (n=9) and Sp4-*null* (n=10) mice were tested for their male sexual behaviour with an estrous female. All males were subjected to a total of 5 tests (once a week) andall behavioural tests were conducted in the male's home cage. The test began when a stimulus female was placed in the male's cage. The latency to the first mount, intromission, and ejaculation, as well as the number of mounts, intromissions, and ejaculations were recorded. The test lasted until ejaculation occurred or 30 minutes if no ejaculation was achieved. Males that ejaculated during one of the five tests were not tested again. After the last test, the males were killed and weighed. Subsequently, the testes and seminal vesicles were dissected and weighed.

Female coital behaviour. Female wild-type (n=8) and Sp4-*null* (n=9) mice were ovariectomized in adulthood using the same procedure as described for the stimulus females. By contrast, the experimental females were not implanted but received two injections of 10 µg estradiol benzoate (EB) 48 and 24h before testing, with an additional progesterone injection of 500 µg 2-4 hours prior to behavioural testing. All lordosis tests were conducted in a Plexiglas aquarium (35 cm long X 25 cm high X 19 cm wide), the floor of which was covered with fresh sawdust. At the beginning of each test, a sexually active male was placed alone in the aquarium and allowed to adapt for 15 min. Subsequently, an experimental female was placed in the aquarium and the lordosis responses of the female to the mounts of the stimulus male were recorded. The test lasted until the female had received 10 mounts or 10 minutes had elapsed. All females were tested a total of three times for lordosis behaviour. At the end of each test, a lordosis quotient (LQ) was calculated by dividing the number of lordotic responses by the number of mounts X 100%. After the last test, the experimental animals received daily injections of estradiol benzoate for 7 days. 24 hours after the last injection the females were killed, the body weights determined and the uteri dissected and weighed.

Analysis of odour preferences of male and female *Sp4*-deficient mice

New groups of male and female *Sp4*$^{-/-}$ and their wild-type littermates were tested for odour preferences in a Y-maze adapted for mice.[16] As in Experiment 1, males were left gonadally intact, whereas females were ovariectomized in adulthood following the procedure as described above. In contrast with Experiment 1, females were implanted with a Silastic capsule containing 17β-estradiol (1:1 dilution with cholesterol). In a pretest, mice were put for 5 minutes in the Y-maze without any stimulus animals placed in the goal boxes, to adapt to the testing apparatus and to determine whether they would develop any side preferences. Then, mice were subjected to a total of two consecutive tests (5 minutes each) in which

they were offered the choice between either volatile body odours from an estrous female and those of an intact male (test 1) or non-volatile odours form bedding soiled by males and that soiled by estrous females (test 2). To expose the subjects to volatile body odours only, stimulus animals were anaesthetized to prevent them from interacting by vocalizations with the experimental subjects, and placed in the distal goal boxes of the Y-maze. Furthermore, the doors of the goal boxes were opaque to prevent the subject from seeing the stimulus animals. To expose the subject to volatile odours, an electric fan placed behind the start box at the beginning of the stem of the Y-maze pulled air over the stimulus animals placed in the distal goal boxes. Furthermore, all doors were perforated to pull air through the entire maze. The time the subject spent sniffing the male and female odours (poking its nose through the holes in the door and/or actively sniffing the door of the goal box) was recorded with a stopwatch. In addition, the number of entries into each arm of the Y-maze was scored. The maze was cleaned with 70% alcohol between trials.

Soiled bedding was collected from stimulus animals (n=3) that were treated with estradiol by silastic capsules and with progesterone by SC injection; they were placed in a cage containing fresh sawdust at the time of the progesterone injection. Bedding was collected 10 hours later. Likewise, gonadally intact males (n=3) were placed in a cage containing fresh sawdust and bedding was collected 10 hours later. All bedding was stored in Ziploc freezer bags at -80°C until used in the experiment. A bowl containing bedding was placed at the end of each arm of the Y-maze. The doors separating the goal boxes from the rest of the maze were removed. The time spent sniffing the bedding was recorded for each subject with a stopwatch. The maze was cleaned with 70% alcohol between tests.

Statistical Analysis

The data on the behaviour tests were compared by analysis of variance (ANOVA). When appropriate, ANOVAs were followed by Tukey highest significance difference (HSD) post hoc comparisons adapted for repeated measures ANOVA. For statistical analysis of the weight measurements, the Student's t-test was performed.

References

1. Philipsen, S. & Suske, G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* **27**, 2991-3000 (1999).
2. Schuh, R. et al. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene. *Cell* **47**, 1025-32 (1986).
3. Gidoni, D. et al. Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. *Science* **230**, 511-7 (1985).
4. Giglioni, B., Comi, P., Ronchi, A., Mantovani, R. & Ottolenghi, S. The same nuclear proteins bind the proximal CACCC box of the human beta-globin promoter and a similar sequence in the enhancer. *Biochem Biophys Res Commun* **164**, 149-55 (1989).
5. Imataka, K. [Side effects and safety in drug therapy of hypertension]. *Nippon Rinsho* **50 Suppl**, 381-7 (1992).
6. Letovsky, J. & Dynan, W. S. Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. *Nucleic Acids Res* **17**, 2639-53 (1989).
7. Suske, G. The Sp-family of transcription factors. *Gene* **238**, 291-300 (1999).
8. Bouwman, P. & Philipsen, S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* **195**, 27-38 (2002).
9. Hagen, G., Muller, S., Beato, M. & Suske, G. Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. *Nucleic Acids Res* **20**, 5519-25 (1992).
10. Gollner, H. et al. Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. *Genes Cells* **6**, 689-97 (2001).
11. Nguyen-Tran, V. T. et al. A novel genetic pathway for sudden cardiac death via defects in the transition

between ventricular and conduction system cell lineages. *Cell* **102**, 671-82 (2000).
12. Supp, D. M., Witte, D. P., Branford, W. W., Smith, E. P. & Potter, S. S. Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility. *Dev Biol* **176**, 284-99 (1996).
13. Van Loo, P. F. et al. Impaired hematopoiesis in mice lacking the transcription factor Sp3. *Blood* **102**, 858-66 (2003).
14. Bouwman, P. et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. *Embo J* **19**, 655-61 (2000).
15. Gollner, H., Dani, C., Phillips, B., Philipsen, S. & Suske, G. Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**, 77-83 (2001).
16. Petrulis, A. & Johnston, R. E. Lesions centered on the medial amygdala impair scent-marking and sex-odor recognition but spare discrimination of individual odors in female golden hamsters. *Behav Neurosci* **113**, 345-57 (1999).
17. Ninomiya, K. & Kimura, T. Male odors that influence the preference of female mice: roles of urinary and preputial factors. *Physiol Behav* **44**, 791-5 (1988).
18. Mossman, C. A. & Drickamer, L. C. Odor preferences of female house mice (Mus domesticus) in seminatural enclosures. *J Comp Psychol* **110**, 131-8 (1996).
19. Gomez, D. M. & Newman, S. W. Differential projections of the anterior and posterior regions of the medial amygdaloid nucleus in the Syrian hamster. *J Comp Neurol* **317**, 195-218 (1992).
20. Scalia, F. & Winans, S. S. The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. *J Comp Neurol* **161**, 31-55 (1975).
21. Korach, K. S. Insights from the study of animals lacking functional estrogen receptor. *Science* **266**, 1524-7 (1994).
22. Lubahn, D. B. et al. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* **90**, 11162-6 (1993).
23. Krege, J. H. et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A* **95**, 15677-82 (1998).
24. Ogawa, S., Taylor, J. A., Lubahn, D. B., Korach, K. S. & Pfaff, D. W. Reversal of sex roles in genetic female mice by disruption of estrogen receptor gene. *Neuroendocrinology* **64**, 467-70 (1996).
25. Korach, K. S. et al. Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res* **51**, 159-86; discussion 186-8 (1996).
26. Sims, N. A. et al. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. *Bone* **30**, 18-25 (2002).
27. Couse, J. F. et al. Disruption of the mouse oestrogen receptor gene: resulting phenotypes and experimental findings. *Biochem Soc Trans* **23**, 929-35 (1995).
28. Balthazart, J., Reid, J., Absil, P., Foidart, A. & Ball, G. F. Appetitive as well as consummatory aspects of male sexual behavior in quail are activated by androgens and estrogens. *Behav Neurosci* **109**, 485-501 (1995).
29. Vagell, M. E. & McGinnis, M. Y. The role of aromatization in the restoration of male rat reproductive behavior. *J Neuroendocrinol* **9**, 415-21 (1997).
30. Baum, M. J. Differentiation of coital behavior in mammals: a comparative analysis. *Neurosci Biobehav Rev* **3**, 265-84 (1979).
31. Keverne, E. B. Importance of olfactory and vomeronasal systems for male sexual function. *Physiol Behav* **83**, 177-87 (2004).
32. Fewell, G. D. & Meredith, M. Experience facilitates vomeronasal and olfactory influence on Fos expression in medial preoptic area during pheromone exposure or mating in male hamsters. *Brain Res* **941**, 91-106 (2002).
33. Pankevich, D. E., Baum, M. J. & Cherry, J. A. Olfactory sex discrimination persists, whereas the preference for urinary odorants from estrous females disappears in male mice after vomeronasal organ removal. *J Neurosci* **24**, 9451-7 (2004).
34. Stowers, L., Holy, T. E., Meister, M., Dulac, C. & Koentges, G. Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science* **295**, 1493-500 (2002).
35. Leypold, B. G. et al. Altered sexual and social behaviors in trp2 mutant mice. *Proc Natl Acad Sci U S A* **99**, 6376-81 (2002).
36. Honda, S., Harada, N., Ito, S., Takagi, Y. & Maeda, S. Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochem Biophys Res Commun* **252**, 445-9 (1998).
37. Bakker, J., Honda, S., Harada, N. & Balthazart, J. Sexual partner preference requires a functional aromatase (cyp19) gene in male mice. *Horm Behav* **42**, 158-71 (2002).
38. Naftolin, F., Ryan, K. J., Davies, I. J., Petro, Z. & Kuhn, M. The formation and metabolism of estrogens in brain tissues. *Adv Biosci* **15**, 105-21 (1975).
39. MacLusky, N. J. & Naftolin, F. Sexual differentiation of the central nervous system. *Science* **211**, 1294-302 (1981).
40. Bakker, J., Honda, S., Harada, N. & Balthazart, J. The aromatase knockout (ArKO) mouse provides new evidence that estrogens are required for the development of the female brain. *Ann N Y Acad Sci* **1007**, 251-62 (2003).
41. Saville, B. et al. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *J Biol Chem* **275**, 5379-87 (2000).
42. Bakker, J., Honda, S., Harada, N. & Balthazart, J. The aromatase knock-out mouse provides new evidence

that estradiol is required during development in the female for the expression of sociosexual behaviors in adulthood. *J Neurosci* **22**, 9104-12 (2002).
43. Kudwa, A. E. & Rissman, E. F. Double oestrogen receptor alpha and beta knockout mice reveal differences in neural oestrogen-mediated progestin receptor induction and female sexual behaviour. *J Neuroendocrinol* **15**, 978-83 (2003).
44. Temple, J. L., Scordalakes, E. M., Bodo, C., Gustafsson, J. A. & Rissman, E. F. Lack of functional estrogen receptor beta gene disrupts pubertal male sexual behavior. *Horm Behav* **44**, 427-34 (2003).
45. deGraffenried, L. A., Hilsenbeck, S. G. & Fuqua, S. A. Sp1 is essential for estrogen receptor alpha gene transcription. *J Steroid Biochem Mol Biol* **82**, 7-18 (2002).
46. deGraffenried, L. A., Hopp, T. A., Valente, A. J., Clark, R. A. & Fuqua, S. A. Regulation of the estrogen receptor alpha minimal promoter by Sp1, USF-1 and ERalpha. *Breast Cancer Res Treat* **85**, 111-20 (2004).
47. Porter, W., Saville, B., Hoivik, D. & Safe, S. Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol Endocrinol* **11**, 1569-80 (1997).
48. Hagen, G., Dennig, J., Preiss, A., Beato, M. & Suske, G. Functional analyses of the transcription factor Sp4 reveal properties distinct from Sp1 and Sp3. *J Biol Chem* **270**, 24989-94 (1995).
49. Hagen, G., Muller, S., Beato, M. & Suske, G. Sp1-mediated transcriptional activation is repressed by Sp3. *Embo J* **13**, 3843-51 (1994).
50. Dennig, J., Beato, M. & Suske, G. An inhibitor domain in Sp3 regulates its glutamine-rich activation domains. *Embo J* **15**, 5659-67 (1996).
51. Li, L., He, S., Sun, J. M. & Davie, J. R. Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* **82**, 460-71 (2004).
52. Schultz, J. R., Petz, L. N. & Nardulli, A. M. Estrogen receptor alpha and Sp1 regulate progesterone receptor gene expression. *Mol Cell Endocrinol* **201**, 165-75 (2003).
53. Kim, K., Thu, N., Saville, B. & Safe, S. Domains of estrogen receptor alpha (ERalpha) required for ERalpha/Sp1-mediated activation of GC-rich promoters by estrogens and antiestrogens in breast cancer cells. *Mol Endocrinol* **17**, 804-17 (2003).
54. Safe, S. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* **62**, 231-52 (2001).
55. Khalyfa, A. et al. Transcription profiling of estrogen target genes in young and old mouse uterus. *Exp Gerontol* **38**, 1087-99 (2003).
56. Klinge, C. M. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res* **29**, 2905-19 (2001).
57. Xie, W., Duan, R., Chen, I., Samudio, I. & Safe, S. Transcriptional activation of thymidylate synthase by 17beta-estradiol in MCF-7 human breast cancer cells. *Endocrinology* **141**, 2439-49 (2000).
58. Marin, M., Karis, A., Visser, P., Grosveld, F. & Philipsen, S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* **89**, 619-28 (1997).
59. Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B. & Cooke, P. S. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A* **97**, 12729-34 (2000).
60. Ohlsson, C. et al. Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice. *Biochem Biophys Res Commun* **278**, 640-5 (2000).
61. Cooke, P. S., Heine, P. A., Taylor, J. A. & Lubahn, D. B. The role of estrogen and estrogen receptor-alpha in male adipose tissue. *Mol Cell Endocrinol* **178**, 147-54 (2001).
62. Sato, T. et al. Late onset of obesity in male androgen receptor-deficient (AR KO) mice. *Biochem Biophys Res Commun* **300**, 167-71 (2003).

CHAPTER 6

Chapter 6

Discussion: concluding remarks and future outlook

Research on the Sp/XKLF factors has revealed that Sp1,[1] Sp3,[2] and Sp4,[3] although sharing high similarity in their protein compositions,[4,5] have distinct biochemical characteristics,[4] and have their own and incomparable contribution to life, as has been demonstrated by gene knockout studies.[3,6-9] However, their precise biological functions are still a timely research topic. In this Chapter, I discuss biological functions of the transcription factors Sp3 and Sp4, on the basis of the data presented in this thesis. An outlook on research that could be performed on Sp3 and Sp4 in the future is given.

What is the physiological role of Sp3?

Studies on the Sp3 protein have shown that complete ablation of the gene encoding this ubiquitously expressed protein causes growth retarded embryos, impaired development of a number of tissues (see references [6,7,10] and Chapter 2), and leads to perinatal death of the fetuses.[6] In principle, pre- and perinatal death might be caused by impaired development of the hematopoietic system.[7,11-14] However, although Sp3 deficiency affects the lymphoid, erythroid, and myeloid compartments of the hematopoietic system, the *Sp3* knockout fetuses experience moderate hematopoietic defects that do not lead to severe anemia with subsequent embryonic death. The hematopoietic defects do not appear to result from an obstruction or blockade in development and differentiation of the *Sp3*$^{-/-}$ hematopoietic precursor, but rather from a reduced efficiency of this process. A delayed progress of hematopoietic differentiation is also observed in the *Sp3*$^{+/-}$ embryos. They have an initial backlog in development of their hematopoietic system compared to wild-type mice, from which they recover within the first weeks after birth. The moderate hematopoietic defects of the Sp3-*null* fetuses argue for the assumption that the presence of Sp3 in the hematopoietic compartments is of benefit, but not a matter of life and death, and suggest that Sp3 is either not an essential transcription factor in this process or that the essential function of the Sp3 protein in hematopoiesis is partially compensated by another factor. Candidate factors for such a counterbalance are the Sp- and Krüppel-like factors that are also expressed in the hematopoietic lineages, namely Sp1 (unpublished results, U. Jägle, 2001), Sp4 (unpublished results, P.F. van Loo and R.H. Hendriks, 2003), Eklf,[11,12] and Bklf.[15] Breeding of knockout alleles of these factors into the *Sp3*$^{+/-}$ background could reveal if one of these factors is partially redundant with Sp3 during hematopoiesis. If a factor is partially redundant, the double heterozygous setting of this factor and Sp3 will exaggerate the hematopoietic phenotype of *Sp3* knockout. Furthermore, as redundancy most probably takes place at the transcriptional level, it will increase the interaction of the compensating Krüppel-like factor to regulatory elements of Sp3 target genes. Such an increase in binding to the DNA could be investigated by chromatin immune precipitation (ChiP) assays[16,17] when Sp3 target genes have been discovered.

The discovery of cardiac malformations in the *Sp3*$^{-/-}$ embryos has elucidated their premature cause of death. A new question that arises is: how would the *Sp3*-deficient mouse develop when it would not encounter cardiac malformations during embryogenesis. Would the *Sp3*$^{-/-}$ mouse still die prematurely or would it survive? Would the impaired tooth- and lung development as

well as the impaired bone ossification turn out to be only transient phenotypes? Would the disturbed expression of the uteroglobin/Clara cell secretory protein (UG/CCSP), amelogenin, ameloblastin, and osteocalcin[6,10] turn out to be only a reflection of delayed development, or are they indeed *bona fide* Sp3 target genes? Whereas conventional mouse knockout approach makes further research on the effect of Sp3 deficiency on maturation impossible, *Sp3*$^{+/-}$ mice may already provide a hint to the answer. We have observed that *Sp3*$^{+/-}$ mice often display a reduced bodyweight and have an increased chance to die early, as 10-15% of them die before 24 weeks of age. Investigation of their cardiac development at E12.5 and E14.5 did not disclose anatomical heart abnormalities, but it is still possible that *Sp3*$^{+/-}$ mice develop cardiac malformations during adulthood, like *Sp4*$^{-/-}$ mice do.[18] Nevertheless, the *Sp3*$^{+/-}$ mouse indicates that reduced levels of Sp3 can give rise to a phenotype at adulthood. The complete absence of Sp3 would therefore be expected to result in a more severe phenotype. To study the effect of complete depletion of *Sp3* on maturation and adulthood, the severe cardiac phenotype has to be circumvented. To this end, advantage could be taken of Cre/lox[19,20] or Flp/FRT[21] recombination systems, and a conditional *Sp3* knockout mouse line should be generated. By tissue-restricted expression of recombinase enzymes, these approaches enable the creation of tissue-restricted ablation of the *Sp3* gene. This would provide a platform to study the effect of Sp3 deficiency on the developmental processes beyond E16.5 and without the influence of disturbed blood circulation, which is caused by cardiac dysfunction. With a conditional *Sp3* knockout by the Cre/lox systems, tooth development in the absence of Sp3 could be analysed by a Cre recombinase under the control of the promoter region of the *mouse dentin sialophosphoprotein* (*Dspp*) gene,[22] whereas lung development in the absence of Sp3 could be investigated by Cre controlled by doxycycline-dependent *surfactant associated protein C* (*Sftpc*) promoter.[23,24] Ossification of the bones of the *Sp3*$^{-/-}$ mice, a process that depends on chondrocytes and osteoblasts cells, could be investigated by a chondrocyte or an osteoblast specific deletion of *Sp3*. A *procollagen type II* (*Col2*) driven Cre[25,26] can deplete *Sp3* form chondrocytes, whereas Cre under the control of the promoter regions of the *bone gamma-carboxyglutamate protein 2* (*Bglap2* or *OG2*) or *procollagen, type I, α 1* (Col1a1) genes[27] produces an osteoblast-specific knockout of *Sp3*. Likewise, depletion of *Sp3* in the heart beyond the remodelling stage by an inducible Cre-Nkx2-5 construct[28] enables investigation of the effect of Sp3 deficiency on the heart during maturation and adulthood. It should be taken into account that several cell types from outside the heart fields contribute to heart development.[29,30] To exclude that the heart phenotype arises from a defect in these cell types, the promoters that are used to create tissue-specific ablation of *Sp3* should not be expressed in these cells.

The heart comprises many different cell types, for example myocytes, epicardial derived cells (EPDCs) and endothelial cells.[31-33] The cells that contribute to the heart arise form the primary and secondary heart fields[34,35] as well as from areas outside the two heart fields.[29,30] As a consequence of the numerous cell types in the heart [31] and the ubiquitous expression of Sp3, it is not clear which cell type(s) contribute(s) to the cardiac phenotype of the *Sp3*$^{-/-}$ fetuses. That it is important to map these cells, shows the r*etinoic acid receptor (Rxrα)* knockout mouse.

The *Rxrα*$^{-/-}$ mice, displaying a cardiac phenotype similar to that of the *Sp3*$^{-/-}$ mouse, has a myocardial phenotype that does not descend form a defect in the myocardial lineage.[36-38] The conditional knockout strategy may help, by targeted ablation of *Sp3* in specific cell types, to trace back the origin of the Sp3-*null* phenotype to specific cell types. Recommended promoters to drive the recombinase enzymes are, for example the *paired box gene 3* (*Pax3*) for neural crest cells,[39,40] the *endothelial-specific receptor tyrosine kinase* (*Tek* or *Tie2*) for the endothelial cell-lineage[41] and *myosin light chain 2v* (*Mlc2v*)[38] or *myosin light chain 2a* (*Mlc2a*) for myocytes.[42-44]

Abnormal electrophysiological characteristics of the heart have been shown to contribute to severe cardiac malformations. For example, mutant mice for the gap junction proteins connexin40, -43, and –45 (Cnx40, -43, -45), proteins required for the propagation of electrical pulse in the heart,[45] display embryonic cardiac morphological abnormalities along with aberrant electrical impulse propagation. The *Cnx40*-deficient mice display double-outlet right ventricle (DORV) or tetralogy of Fallot (TOF),[46] and exhibit conduction abnormalities,[47] whereas the Cnx43-*null* mice display abnormal coronary artery patterns[48] along with slow propagation of ventricular activation and sudden arrhythmic death.[49,50] The *Cnx45*$^{-/-}$ mice develop abnormal cushions, have a block in contraction and die before E10.5.[51,52] The necessity of proper pulse propagation for heart development, but also the probability of aberrant electrophysiological characteristics due to the hypoplasticity of the myocardium of the *Sp3*$^{-/-}$ hearts itself,[53] argue for an assessment of an electrocardiogram (ECG) of the *Sp3*$^{-/-}$ fetuses. If the early perinatal death of the Sp3-*null* embryos makes it impossible to obtain an ECG, a transgenic mouse approach with a reporter gene under the control of the *Cnx40, -43 or -45* promoter regions[54] may reveal if these gap junction proteins have a disturbed expression pattern in the Sp3-*null* mice and reveal an underlying aberrant pulse propagation.

The possibility that the cardiac phenotype of the *Sp3*$^{-/-}$ mice has its onset during the early phase of heart development has to be considered, as an early morphological abnormality in the heart can develop into severe cardiac malformations and cause prenatal death. This has been shown for an atrium-specific deletion of the *Mlc2a* gene.[55] That a local cardiac abnormality leads to severe overall cardiac malformations may be the result of shear stress, as a number of previous studies have demonstrated that alterations in fluid forces experienced by endothelial cells, including those of the endocardium, can result in changes in their alignment and changes in gene expression.[56-60] Therefore the effect of Sp3 deficiency on the earlier phases of the heart development, between the staged of the fusion of the cardiac crescent into a linear tube and the primary looping of the heart tube,[61] has to be examined in detail in the Sp3-*null* embryos.

What is the physiological role of Sp4?

Deficiency of the transcription factor Sp4 in the mouse causes a versatile phenotype. One of the most obvious abnormalities of the phenotype is the impaired reproduction of the male and the female sex. Studies on the coital behaviour of both sexes have revealed

that their impaired reproduction is, at least, brought about by the absence of normal sexual behaviour to conspecifics. Coital behaviour is induced and coordinated by the brain, which is also a major site of Sp4 expression.[3,9] However, as the precise expression pattern of Sp4 in the brain has not been analysed in detail yet, it is impossible to link the expression pattern of Sp4 with the impaired coital behaviour of the *Sp4*$^{-/-}$ male and female mice. This requires a detailed examination of the expression of the Sp4 protein in the brain tissue by immunohistochemistry.

The brain compartments that normally express Sp4 might have an abnormal anatomical appearance in the *Sp4*$^{-/-}$ mice as a consequence of the lack of Sp4. When the Sp4 expressing brain compartments have been localized via an immunohistochemistry approach, these structures should be examined for anatomical appearance in *Sp4*$^{-/-}$ male and female mice. If abnormal organization of one of these structures can be identified, this might shed light on the abnormal coital behaviour of these animals. For this inquiry, advantage should be taken of the *β-galactosidase* gene that has been knocked in the Sp4 locus. Expression of the *β-galactosidase* gene is regulated by the endogenous Sp4 promoter, and expression of the *lacZ* gene in heterozygous mice should mirror the structures that express Sp4 in the wild-type situation. Comparison with *lacZ* expression in the knockouts should reveal any structures that are affected by the absence of Sp4.

The infertility of male *Sp4*-deficeint mice is primarily the result of absence of sexual behaviour. Normally, coital behaviour is triggered by volatile and non-volatile odours of conspecifics. However, this process is impaired in *Sp4*-deficient males. They show a strongly reduced interest in volatile and non-volatile odours, and they lack preference for non-volatile odours of an estrous female over such odours from a gonadally intact male. In rodents, non-volatile odours of conspecifics are detected and processed by the polysynaptic accessory olfactory system (AOS).[62-64] The olfactory cues are first detected by the vomeronasal organ (VNO)[65,66] via the Vr1[67] and Vr2 receptors.[68,69] When these receptors are exposed to olfactory cues, the VNO project these stimuli into the accessory olfactory bulb (AOB).[64] Subsequently, stimuli are projected along various neural pathways, including the anterior-dorsal medial amygdale (MeAD) and the posterior-doral medial amygdale (MePD).[70,71]

Possibly, the projection of these olfactory cues along the neural pathways is impaired in the brain of the male *Sp4*$^{-/-}$ mouse. This hypothesis could be addressed by an analysis of the activation of the downstream neuronal pathways in the brain of *Sp4*$^{-/-}$ males after they have been in contact with odours of a male or estrous female. A reflection of neuronal activity is the expression of c-Fos. Fos protein becomes transiently expressed in neurons in response to direct stimulation by growth factors ad neurotransmitters,[72-74] and it is known that several areas of the AOS in the male rodent brain display an increase in Fos expression after an exposure to female odours.[75,76] Thus, by taking advantage of Fos expression, examination of this expression by immunohistochemistry might reveal a block in the functioning of the AOS of the Sp4-*null*.

How do Sp3 and Sp4 mediate transcription?

The Sp factors are likely to function at the level of transcriptional regulation, as they are transcription factors. This implies that the phenotypes displayed by their knockout mouse lines may primarily be caused by aberrant transcription of specific target genes. The discovery of these target genes is therefore absolutely essential to unravel the puzzle.

Previous studies on the *Sp3*$^{-/-}$ mouse revealed a number of genes that have an altered expression in the absence of Sp3: *Ug/Ccsp; amelogenin; ameloblastin; osteocalcin,*[6,10] and *Carp* (this thesis). Furthermore, previous attempts to identify target genes of Sp3 by a cDNA microarray approach or a method to specifically amplify differentially expressed genes (the selective amplification via biotin- and restriction-mediated enrichment/SABRE method)[77] on whole E13.5 *Sp3*$^{-/-}$ embryos were unsuccessful. The microarray experiments described in this thesis (Chapter 4) also turned out to be not that enlightening. Although this experiment revealed a number of genes that might be differentially expressed in the absence of Sp3, the placing of these genes in the framework of the *Sp3*$^{-/-}$ embryonic cardiac phenotype would be based on speculation, due to the limited available data on their expression patterns, biochemical characteristics, and *in vivo* functions. This conclusion forces us to validation the outcome of these microarray experiments, but also to study the functions of these genes in more detail. To identify Sp3 target genes among these genes, their regulatory regions have to be examined for the presence of functional Sp binding sites[78] and CACC-boxes,[15] which are both target sequences for Sp factors. Such analysis could be done by gel retardation assays[79] or ChiP experiments.[16]

It cannot be excluded that the few and partially unclear data from the microarray experiments described in Chapter 4 were caused by technical shortcomings. This argues for a repeat of these microarray experiments. Furthermore, as the cardiac phenotype of the *Sp3*$^{-/-}$ mice may have its onset during very early developmental stages, the gene expression patterns of the primitive Sp3-*null* hearts should be analysed by a microarray approach.

Nevertheless, the limited progress that has been made with the microarrays and SABRE experiments in the discovery of differentially expressed genes suggests that other approaches have to be chosen to unravel the Sp3 puzzle. A new approach to find Sp3 target genes could be the identification of the regulatory elements that bind Sp3. This can be achieved by a pull down of these regulatory elements with an antibody against Sp3 or a tagged Sp3 protein, and to analyse them by subsequent cloning and sequencing steps.[17,80,81] This method can also be applied to the Sp4 protein, in order to elucidate its biological function.

The ubiquitous expression pattern of Sp3 suggests that the factor fulfils a general and identical function in all cell types. However, it is also possible that Sp3, despite its ubiquitous expression, fulfils a unique function in each cell type as a transcriptional mediator. To test this hypothesis, Sp3 target genes should be identified in at least two different tissues, by the earlier proposed analysis methods, and the data obtained from both tissues should be compared. Suitable candidate tissues for such a study are the heart and hematopoietic system, as both tissues are affected in the *Sp3*$^{-/-}$ mouse.

There are, similar to the situation for Sp3, no target genes of Sp4 identified via analysis of the Sp4 knockout mouse line. However, the uterus might be a good candidate tissue to for search Sp4 target genes. The uterus has a disturbed physiological function in the absence of Sp4. In the normal situation, cells of the uterus proliferate and differentiate in response to estrogens. In contrast, the uteri of *Sp4*$^{-/-}$ females display a severely reduced proliferation and differentiation response. This abnormality makes it possible to investigate the abnormal gene expression profile in the *Sp4*-deficient uterus upon estrogen treatment by comparing this profile with the gene expression profile from a wild-type female uterus that has been treated with estrogen as well. For this study, wild-type and *Sp4* knockout females should be ovariectomized and treated with estrogen for multiple time periods. The treatment of the mice for a number of time periods makes it possible to study the effect that Sp4 depletion has in successive phases of estrogen induction of the uterus. A recommended technique for analysis of the gene expression profiles is the micro-array technique. Target genes might be related to the estrogen signalling, as the uterus anomaly of the *Sp4*$^{-/-}$ females also has been found in the estrogen receptors α and β-*null* mice *(ERα*$^{-/-}$ and *ERβ*$^{-/-}$).[82,83] In these mice, estrogen signalling is disrupted by depletion by one of the estrogen receptors, which gives rise to an impaired proliferation and differentiation of the uterus. Other hints that the Sp4 target genes in the uterus are part of the ER signalling come from the many studies that have shown that Sp1 and Sp3 *in vitro* regulate either the expression of ER itself[84-87] or both the ER,s and target genes.[88-92] Since it is known that the zinc-finger regions of Sp1, Sp3 and Sp4 recognize the GC- and GT-boxes with indistinguishable affinity,[78] it is tempting to hypothesize that Sp4 might also fulfill a function in regulation of the ER cascade.

For the complete understanding of the role of Sp3 and Sp4, not only biological processes and genes that are dependent on Sp3 or Sp4 have to be discovered. It is also necessary that their biochemical characteristics are entirely disclosed and understood. Additional research topics on Sp3 include: what is the contribution of each of the four isoforms of Sp3 to the transcription of Sp3 target genes;[93] how does the inhibitory domain regulate transcription; and how is the activity of this domain modulated.[94,95] Furthermore, the proteins that interact with Sp3 should be discovered and the contribution of the promoter composition to transcriptional potential of Sp3 has to be elucidated.[5]

In conclusion, although the puzzles of Sp3 and Sp4 are not finished yet, more puzzle pieces have been put in place during the last few years. The puzzles display a picture of the physiological processes that are depending on Sp3 and Sp4, and of their target genes. Building the complete picture is the challenge faced by ongoing research on these Sp-factors.

References

1. Kadonaga, J. T., Carner, K. R., Masiarz, F. R. & Tjian, R. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**, 1079-90 (1987).
2. Kingsley, C. & Winoto, A. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. *Mol Cell Biol* **12**, 4251-61 (1992).
3. Supp, D. M., Witte, D. P., Branford, W. W., Smith, E. P. & Potter, S. S. Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility. *Dev Biol* **176**, 284-99 (1996).
4. Bouwman, P. & Philipsen, S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* **195**, 27-38 (2002).
5. Suske, G. The Sp-family of transcription factors. *Gene* **238**, 291-300 (1999).
6. Bouwman, P. et al. Transcription factor Sp3 is essential for post-natal survival and late tooth development. *Embo J* **19**, 655-61 (2000).
7. Van Loo, P. F. et al. Impaired hematopoiesis in mice lacking the transcription factor Sp3. *Blood* **102**, 858-66 (2003).
8. Marin, M., Karis, A., Visser, P., Grosveld, F. & Philipsen, S. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. *Cell* **89**, 619-28 (1997).
9. Gollner, H. et al. Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. *Genes Cells* **6**, 689-97 (2001).
10. Gollner, H., Dani, C., Phillips, B., Philipsen, S. & Suske, G. Impaired ossification in mice lacking the transcription factor Sp3. *Mech Dev* **106**, 77-83 (2001).
11. Perkins, A. C., Sharpe, A. H. & Orkin, S. H. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature* **375**, 318-22 (1995).
12. Nuez, B., Michalovich, D., Bygrave, A., Ploemacher, R. & Grosveld, F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature* **375**, 316-8 (1995).
13. Pevny, L. et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**, 257-60 (1991).
14. Tsai, F. Y. & Orkin, S. H. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* **89**, 3636-43 (1997).
15. Crossley, M. et al. Isolation and characterization of the cDNA encoding BKLF/TEF-2, a major CACCC-box-binding protein in erythroid cells and selected other cells. *Mol Cell Biol* **16**, 1695-705 (1996).
16. Gould, A. P., Brookman, J. J., Strutt, D. I. & White, R. A. Targets of homeotic gene control in Drosophila. *Nature* **348**, 308-12 (1990).
17. Javed, A. et al. Chromatin immunoprecipitation. *Methods Mol Biol* **285**, 41-4 (2004).
18. Nguyen-Tran, V. T. et al. A novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages. *Cell* **102**, 671-82 (2000).
19. Sauer, B. Inducible gene targeting in mice using the Cre/lox system. *Methods* **14**, 381-92 (1998).
20. Sauer, B. & Henderson, N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* **85**, 5166-70 (1988).
21. Vooijs, M., van der Valk, M., te Riele, H. & Berns, A. Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. *Oncogene* **17**, 1-12 (1998).
22. Sreenath, T. L., Cho, A., Thyagarajan, T. & Kulkarni, A. B. Odontoblast-specific expression of cre recombinase successfully deletes gene segments flanked by loxP sites in mouse teeth. *Genesis* **35**, 94-9 (2003).
23. Perl, A. K., Tichelaar, J. W. & Whitsett, J. A. Conditional gene expression in the respiratory epithelium of the mouse. *Transgenic Res* **11**, 21-9 (2002).
24. Perl, A. K., Wert, S. E., Nagy, A., Lobe, C. G. & Whitsett, J. A. Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc Natl Acad Sci U S A* **99**, 10482-7 (2002).
25. Terpstra, L. et al. Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. *J Cell Biol* **162**, 139-48 (2003).
26. Ovchinnikov, D. A., Deng, J. M., Ogunrinu, G. & Behringer, R. R. Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis* **26**, 145-6 (2000).
27. Castro, C. H. et al. Development of mice with osteoblast-specific connexin43 gene deletion. *Cell Commun Adhes* **10**, 445-50 (2003).
28. Moses, K. A., DeMayo, F., Braun, R. M., Reecy, J. L. & Schwartz, R. J. Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. *Genesis* **31**, 176-80 (2001).
29. Wessels, A. & Perez-Pomares, J. M. The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. *Anat Rec* **276A**, 43-57 (2004).
30. Sieber-Blum, M. Cardiac neural crest stem cells. *Anat Rec* **276A**, 34-42 (2004).
31. Fishman, M. C. & Chien, K. R. Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-117 (1997).
32. Gittenberger-de Groot, A. C., Vrancken Peeters, M. P., Bergwerff, M., Mentink, M. M. & Poelmann, R. E. Epicardial outgrowth inhibition leads to compensatory mesothelial outflow tract collar and abnormal cardiac septation and coronary formation. *Circ Res* **87**, 969-71 (2000).
33. Gittenberger-de Groot, A. C., Vrancken Peeters, M. P., Mentink, M. M., Gourdie, R. G. & Poelmann,

R. E. Epicardium-derived cells contribute a novel population to the myocardial wall and the atrioventricular cushions. *Circ Res* **82**, 1043-52 (1998).
34. Yutzey, K. E. & Kirby, M. L. Wherefore heart thou? Embryonic origins of cardiogenic mesoderm. *Dev Dyn* **223**, 307-20 (2002).
35. Kelly, R. G. & Buckingham, M. E. The anterior heart-forming field: voyage to the arterial pole of the heart. *Trends Genet* **18**, 210-6 (2002).
36. Tran, C. M. & Sucov, H. M. The RXRalpha gene functions in a non-cell-autonomous manner during mouse cardiac morphogenesis. *Development* **125**, 1951-6 (1998).
37. Kubalak, S. W., Hutson, D. R., Scott, K. K. & Shannon, R. A. Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos. *Development* **129**, 733-46 (2002).
38. Chen, J., Kubalak, S. W. & Chien, K. R. Ventricular muscle-restricted targeting of the RXRalpha gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis. *Development* **125**, 1943-9 (1998).
39. Epstein, J. A. et al. Migration of cardiac neural crest cells in Splotch embryos. *Development* **127**, 1869-78 (2000).
40. Li, J., Chen, F. & Epstein, J. A. Neural crest expression of Cre recombinase directed by the proximal Pax3 promoter in transgenic mice. *Genesis* **26**, 162-4 (2000).
41. Kisanuki, Y. Y. et al. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* **230**, 230-42 (2001).
42. Lee, K. J. et al. Myosin light chain-2 luciferase transgenic mice reveal distinct regulatory programs for cardiac and skeletal muscle-specific expression of a single contractile protein gene. *J Biol Chem* **267**, 15875-85 (1992).
43. O'Brien, T. X., Lee, K. J. & Chien, K. R. Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc Natl Acad Sci U S A* **90**, 5157-61 (1993).
44. Kubalak, S. W., Miller-Hance, W. C., O'Brien, T. X., Dyson, E. & Chien, K. R. Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *J Biol Chem* **269**, 16961-70 (1994).
45. Gros, D. et al. Genetically modified mice: tools to decode the functions of connexins in the heart-new models for cardiovascular research. *Cardiovasc Res* **62**, 299-308 (2004).
46. Gu, H., Smith, F. C., Taffet, S. M. & Delmar, M. High incidence of cardiac malformations in connexin40-deficient mice. *Circ Res* **93**, 201-6 (2003).
47. VanderBrink, B. A. et al. Connexin40-deficient mice exhibit atrioventricular nodal and infra-Hisian conduction abnormalities. *J Cardiovasc Electrophysiol* **11**, 1270-6 (2000).
48. Li, W. E. et al. An essential role for connexin43 gap junctions in mouse coronary artery development. *Development* **129**, 2031-42 (2002).
49. Vaidya, D. et al. Null mutation of connexin43 causes slow propagation of ventricular activation in the late stages of mouse embryonic development. *Circ Res* **88**, 1196-202 (2001).
50. Gutstein, D. E. et al. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. *Circ Res* **88**, 333-9 (2001).
51. Nishii, K., Kumai, M. & Shibata, Y. Regulation of the epithelial-mesenchymal transformation through gap junction channels in heart development. *Trends Cardiovasc Med* **11**, 213-8 (2001).
52. Kumai, M. et al. Loss of connexin45 causes a cushion defect in early cardiogenesis. *Development* **127**, 3501-12 (2000).
53. Pashmforoush, M. et al. Nkx2-5 pathways and congenital heart disease; loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. *Cell* **117**, 373-86 (2004).
54. Miquerol, L. et al. Architectural and functional asymmetry of the His-Purkinje system of the murine heart. *Cardiovasc Res* **63**, 77-86 (2004).
55. Huang, C. et al. Embryonic atrial function is essential for mouse embryogenesis, cardiac morphogenesis and angiogenesis. *Development* **130**, 6111-9 (2003).
56. Icardo, J. M. Endocardial cell arrangement: role of hemodynamics. *Anat Rec* **225**, 150-5 (1989).
57. Resnick, N., Yahav, H., Schubert, S., Wolfovitz, E. & Shay, A. Signalling pathways in vascular endothelium activated by shear stress: relevance to atherosclerosis. *Curr Opin Lipidol* **11**, 167-77 (2000).
58. Shyy, J. Y. & Chien, S. Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* **91**, 769-75 (2002).
59. Topper, J. N. & Gimbrone, M. A., Jr. Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Mol Med Today* **5**, 40-6 (1999).
60. Gimbrone, M. A., Jr., Topper, J. N., Nagel, T., Anderson, K. R. & Garcia-Cardena, G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci* **902**, 230-9; discussion 239-40 (2000).
61. Fishman, M. C. & Olson, E. N. Parsing the heart: genetic modules for organ assembly. *Cell* **91**, 153-6 (1997).
62. Gomez, D. M. & Newman, S. W. Differential projections of the anterior and posterior regions of the medial amygdaloid nucleus in the Syrian hamster. *J Comp Neurol* **317**, 195-218 (1992).
63. Halpern, M. The organization and function of the vomeronasal system. *Annu Rev Neurosci* **10**, 325-62 (1987).
64. Scalia, F. & Winans, S. S. The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. *J Comp Neurol* **161**, 31-55 (1975).
65. Buck, L. B. Unraveling chemosensory diversity. *Cell* **83**, 349-52 (1995).

66. Rodriguez, I., Feinstein, P. & Mombaerts, P. Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell* **97**, 199-208 (1999).
67. Dulac, C. & Axel, R. A novel family of genes encoding putative pheromone receptors in mammals. *Cell* **83**, 195-206 (1995).
68. Matsunami, H. & Buck, L. B. A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* **90**, 775-84 (1997).
69. Herrada, G. & Dulac, C. A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* **90**, 763-73 (1997).
70. Maragos, W. F., Newman, S. W., Lehman, M. N. & Powers, J. B. Neurons of origin and fiber trajectory of amygdalofugal projections to the medial preoptic area in Syrian hamsters. *J Comp Neurol* **280**, 59-71 (1989).
71. Lehman, M. N. & Winans, S. S. Evidence for a ventral non-strial pathway from the amygdala to the bed nucleus of the stria terminalis in the male golden hamster. *Brain Res* **268**, 139-46 (1983).
72. Williams, S., Evan, G. I. & Hunt, S. P. Changing patterns of c-fos induction in spinal neurons following thermal cutaneous stimulation in the rat. *Neuroscience* **36**, 73-81 (1990).
73. Hunt, S. P., Pini, A. & Evan, G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* **328**, 632-4 (1987).
74. Sagar, S. M., Sharp, F. R. & Curran, T. Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* **240**, 1328-31 (1988).
75. Paredes, R. G., Lopez, M. E. & Baum, M. J. Testosterone augments neuronal Fos responses to estrous odors throughout the vomeronasal projection pathway of gonadectomized male and female rats. *Horm Behav* **33**, 48-57 (1998).
76. Bressler, S. C. & Baum, M. J. Sex comparison of neuronal Fos immunoreactivity in the rat vomeronasal projection circuit after chemosensory stimulation. *Neuroscience* **71**, 1063-72 (1996).
77. Lavery, D. J., Lopez-Molina, L., Fleury-Olela, F. & Schibler, U. Selective amplification via biotin- and restriction-mediated enrichment (SABRE), a novel selective amplification procedure for detection of differentially expressed mRNAs. *Proc Natl Acad Sci U S A* **94**, 6831-6 (1997).
78. Philipsen, S. & Suske, G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* **27**, 2991-3000 (1999).
79. Perelygina, L. M., Tomilin, N. V. & Podgornaya, O. I. Alu-family repeat binding protein from HeLa cells which interacts with regulatory region of SV40 virus genome. *Mol Biol Rep* **12**, 111-6 (1987).
80. Phelps, D. E. & Dressler, G. R. Identification of novel Pax-2 binding sites by chromatin precipitation. *J Biol Chem* **271**, 7978-85 (1996).
81. de Boer, E. et al. Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice. *Proc Natl Acad Sci U S A* **100**, 7480-5 (2003).
82. Lubahn, D. B. et al. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* **90**, 11162-6 (1993).
83. Krege, J. H. et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A* **95**, 15677-82 (1998).
84. deGraffenried, L. A., Hilsenbeck, S. G. & Fuqua, S. A. Sp1 is essential for estrogen receptor alpha gene transcription. *J Steroid Biochem Mol Biol* **82**, 7-18 (2002).
85. deGraffenried, L. A., Hopp, T. A., Valente, A. J., Clark, R. A. & Fuqua, S. A. Regulation of the estrogen receptor alpha minimal promoter by Sp1, USF-1 and ERalpha. *Breast Cancer Res Treat* **85**, 111-20 (2004).
86. Porter, W., Saville, B., Hoivik, D. & Safe, S. Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol Endocrinol* **11**, 1569-80 (1997).
87. Saville, B. et al. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *J Biol Chem* **275**, 5379-87 (2000).
88. Li, L., He, S., Sun, J. M. & Davie, J. R. Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* **82**, 460-71 (2004).
89. Kim, K., Thu, N., Saville, B. & Safe, S. Domains of estrogen receptor alpha (ERalpha) required for ERalpha/Sp1-mediated activation of GC-rich promoters by estrogens and antiestrogens in breast cancer cells. *Mol Endocrinol* **17**, 804-17 (2003).
90. Khalyfa, A. et al. Transcription profiling of estrogen target genes in young and old mouse uterus. *Exp Gerontol* **38**, 1087-99 (2003).
91. Safe, S. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* **62**, 231-52 (2001).
92. Schultz, J. R., Petz, L. N. & Nardulli, A. M. Estrogen receptor alpha and Sp1 regulate progesterone receptor gene expression. *Mol Cell Endocrinol* **201**, 165-75 (2003).
93. Sapetschnig, A., Koch, F., Rischitor, G., Mennenga, T. & Suske, G. Complexity of translationally controlled transcription factor Sp3 isoform expression. *J Biol Chem* (2004).
94. Ross, S., Best, J. L., Zon, L. I. & Gill, G. SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell* **10**, 831-42 (2002).
95. Sapetschnig, A. et al. Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *Embo J* **21**, 5206-15 (2002).

Samenvatting

Een organisme is opgebouwd uit vele verschillende celtypes, die ieder hun eigen biologische functie vervullen. Alleen wanneer de cellen hun functie goed uitvoeren, kan het organisme waartoe ze behoren zich ontwikkelen en instandhouden. Welke functie een cel heeft en hoe deze moet worden uitgevoerd staat gecodeerd in het erfelijk materiaal van de cel, het DNA. Al het DNA samen heet het genoom. Het genoom bevat de genen die coderen voor eiwitten, dit zijn de uitvoerders van de processen in de cel. De combinatie van cellulaire processen in een cel geeft de cel haar uiteindelijke functie in het organisme. Voor de vorming van eiwitten wordt de DNA-code van een gen allereerst via transcriptie overgeschreven in een RNA-molecuul. Dit RNA-molecuul wordt vervolgens via translatie vertaald in een keten van aminozuren, dit is het eiwit. Deze twee processen zijn strikt gereguleerd, zodat de productie van de eiwitten op de juiste plaats en op het juiste moment plaatsvindt. Het eerste proces, de transcriptie, wordt gereguleerd door transcriptiefactoren. Dit zijn eiwitten die kunnen binden aan specifieke DNA-motieven in het genoom. Door binding aan dergelijke DNA-motieven kunnen deze factoren de transcriptie van een gen stimuleren of juist onderdrukken en zo de aanmaak van een eiwit reguleren. In de cel zijn vele verschillende transcriptiefactoren aanwezig met ieder hun eigen functie en hun eigen voorkeur voor een DNA-motief. Doordat ieder gen beschikt over een unieke combinatie van DNA-motieven die een specifieke combinatie van transcriptiefactoren bindt, kan ieder gen op het juiste moment en op de juiste plaats overgeschreven worden in RNA dat vervolgens weer kan worden vertaald in eiwit. Zo is het mogelijk om vele verschillende celtypen te hebben in één organisme met ieder hun eigen biologische processen en functie.

Dit proefschrift beschrijft de experimenten die zijn uitgevoerd om de functie van Sp3 en Sp4, twee transcriptiefactoren, te begrijpen. Dit is gedaan met behulp van muizenlijnen waarin de genen van Sp3 of Sp4 gericht waren uitgeschakeld. Door deze gerichte uitschakeling konden processen worden geïdentificeerd die Sp3 of Sp4 nodig hebben om goed te verlopen, doordat deze processen juist verstoord waren in de *Sp3*- of *Sp4*-deficiënte muizen. Door middel van het analyseren van deze verstoorde processen kunnen hun biologische functies nu beter worden begrepen.

In *hoofdstuk 1* wordt een overzicht gegeven van de Sp/XKLF (specificity protein/ x Krüppel-like factor) familie van transcriptiefactoren, waartoe Sp3 en Sp4 behoren. In meer detail wordt de Sp subfamilie van de Sp/XKLF familie besproken, die bestaat uit negen Sp factoren (Sp1-9). Onder meer passeren de domeinen die deze Sp factoren gemeenschappelijk hebben de revue, maar ook domeinen die uniek zijn voor één Sp factor. Ingegaan wordt op de invloed die elk domein heeft op de potentie van de Sp factor om als transcriptiefactor het transcriptieproces te reguleren en hoe deze potentie door de cel kan worden gemodelleerd en gereguleerd. Het overzicht van de fenotypes van de bestaande muizenlijnen die deficiënt zijn voor een Sp factor laat zien dat elke Sp factor een unieke biologische functie vervult in embryogenese, postnatale ontwikkeling en overleving.

Analyse van de *Sp3*-deficiënte muis heeft duidelijk gemaakt dat deze transcriptiefactor essentieel is voor de ontwikkeling van een aantal weefsels tijdens de embryogenese, maar ook voor de postnatale overleving van de muis. Echter de doodsoorzaak van *Sp3*$^{-/-}$ muizen kon nog niet worden opgehelderd. *Hoofdstuk 2* beschrijft de bestudering van de ontwikkeling van het bloedvormende weefsel, het hematopoietische systeem. Dit weefsel is bestudeerd omdat Sp3 in de literatuur herhaaldelijk functioneel geassocieerd is met dit weefsel en een defect in het bloedvormende weefsel zou de vroegtijdige dood van *Sp3*$^{-/-}$ muizen kunnen veroorzaken. Analyse van de populatie witte bloedcellen, de lymfocyten (bestaande uit B en T cellen), op dag 18.5 van de embryonale ontwikkeling (E18.5) onthulde een gedeeltelijke blokkade in de T-cel ontwikkeling op het DP ($CD^{4+}CD^{8+}$ cellen) stadium in de *Sp3*$^{-/-}$ zwezerik en een gereduceerde $B220^{+}$ B-cel populatie in de *Sp3*$^{-/-}$ lever en milt. De reductie van deze populatie, die voornamelijk op het conto komt van de $B220^{+}IgM^{+}$ cellen, wordt niet waargenomen als *Sp3*$^{-/-}$ pre-B cellen zich in een kunstmatige experimentele setting (een *in vitro* essay) ontwikkelen tot $B220^{+}IgM^{+}$ cellen. Op E14.5 is tevens het percentage van circulerende foetale rode bloedcellen, de erythrocyten, in de bloedbaan van *Sp3*$^{-/-}$ foetussen sterk verminderd. Dit gaat samen met een verminderde voortgang van de ontwikkeling van foetale *Sp3*$^{-/-}$ voorlopercellen van de erythrocyten tot rijpe erythrocyten in een *in vitro* essay. De vroegtijdige dood van de *Sp3*$^{-/-}$ muizen dwong ons, om voor het bestuderen van de volwassen hematopoiese, een *in vivo* transplantatie experiment uit te voeren. In dit experiment is gekeken of E12.5 *Sp3*$^{-/-}$ levercellen, wanneer deze getransplanteerd werden in muizen waarvan het eigen hematopoietische systeem geinactiveerd was, konden bijdragen aan de vorming van het hematopoietische systeem van de muis waarin ze getransplanteerd werden. De resultaten lieten zien dat de embryonale *Sp3*-deficiënte levercellen in beperkte mate instaat zijn om aan de B- en T-cel populaties bij te dragen, maar niet aan de myeloïde- en erythroïde celpopulaties.

De gevonden afwijkingen aan het hematopoietische systeem kunnen echter niet de vroegtijdige dood van de *Sp3*$^{-/-}$ muizen verklaren. Door nieuwe waarnemingen kregen we de hint dat hartfalen de oorzaak van hun vroegtijdige dood zou kunnen zijn. Zo was waargenomen dat *Sp3*-deficiënte muizen in de C57Bl/6 genetische achtergrond al tijdens de zwangerschap overleden en dat zij daarnaast onderhuids vocht ophoopten op E14.5, wat wijst op een verminderde bloedcirculatie. Deze waarnemingen vormden de aanleiding tot een studie naar hartontwikkeling in *Sp3*$^{-/-}$ foetussen, waarvan de resultaten staan beschreven in *hoofdstuk 3*. Dat de hartontwikkeling van *Sp3*$^{-/-}$ foetussen is verstoord blijkt reeds uit de wijde binnenbocht van hun gedraaide hartbuis op dag E10.5. Deze vroege hartafwijking heeft tot gevolg dat de in- en uitstroomgebieden van het hart zich tijdens de hartontwikkeling niet goed positioneren in het hart. Dit heeft uiteindelijk tot consequentie dat ongeveer 90% van de E14.5 *Sp3*$^{-/-}$ embryo's een hart met een dubbele uitstroom rechterventrikel hebben en dat bijna 65% van hen daarnaast ook nog een dubbele instroom linkerventrikel ontwikkelt. Verder is het myocardium, het spierweefsel van een hart, heel dun en sponzig van structuur in een *Sp3*-deficiënt hart. Dit leidt in meer dan de helft van de Sp3 deficiënte muizen tot gaten in de myocardlaag van de atria en ventrikels, waardoor bloed zich kan ophopen tussen het myocardium en het epicardium, de buitenste laag van het hart. Voor het in kaart

brengen van de rol van Sp3 in de hartontwikkeling hebben we de transcriptie van een aantal hartspecifieke genen, waaronder Nkx2-5, Gata4, Anf en Carp, geanalyseerd in *Sp3*$^{-/-}$ harten van E10.5, E12.5 en E14.5 embryo's. De expressie van de meeste genen is niet veranderd tijdens de ontwikkeling van de *Sp3*$^{-/-}$ harten, met het *Carp* (*cardiac ankyrin repeat protein*) gen als uitzondering. Het Carp transcript is in E14.5 oude *Sp3*$^{-/-}$ harten bijna geheel afwezig. Analyse van de *Carp* promotor maakte duidelijk dat Sp3 kan binden aan DNA-motieven in deze promotor, wat suggereert dat Carp *in vivo* direct door Sp3 wordt gereguleerd.

Het ontstaan van de hartafwijkingen in de *Sp3*$^{-/-}$ foetussen kan echter niet alleen verklaard worden door de sterk verminderde Carp expressie op E14.5. Dit impliceert dat Sp3 daarnaast ook de expressie reguleert van andere genen die belangrijk zijn voor hartontwikkeling. Om deze genen te identificeren zijn er microarray-experimenten uitgevoerd. Deze experimenten, beschreven in *hoofdstuk 4*, boden de mogelijkheid om juist die genen te ontdekken die een afwijkende expressie hebben in de harten van *Sp3*$^{-/-}$ embryo's. Voor de uitvoering van de experimenten zijn de genexpressiepatronen van *Sp3*$^{+/+}$ harten vergeleken met die van *Sp3*$^{-/-}$ harten op de embryonale leeftijden E12.5 en E14.5. De gegenereerde datafiles van de microarray-experimenten zijn vervolgens genormaliseerd en geanalyseerd verschillende statistische procedures. Ondanks dat de kleurinversie experimenten geen duidelijk overlappend expressie patroon hebben en ondanks de negatieve resultaten van de clustering, beide veroorzaakt door beperkte consistentie van de microarray-data als gevolg van technische onvolkomenheden, konden elf mRNA sequenties worden geïdentificeerd die een verlaagde expressie laten zien in E12.5 en E14.5 *Sp3*$^{-/-}$ experimenten. Zes van deze transcripten zijn mogelijk samen met Sp3 betrokken bij de ontwikkeling van het hart en vijf van deze zes transcripten zijn kandidaat targetgenen van Sp3.

Het *Sp4* gen heeft een zeer afgebakend expressie patroon en komt met name tot expressie in de hersenen en zenuwen. In het oog springende afwijkingen van de *Sp4*-deficiënte muizenlijn zijn het ontbreken van paringsgedrag bij de mannetjes en een verstoorde seksueleontwikkeling bij de vrouwtjes. Aangezien de oorzaak van deze afwijkingen nog niet in kaart is gebracht, zijn er aanvullende experimenten uitgevoerd om het primaire defect dat deze afwijkingen veroorzaakt te lokaliseren. Deze experimenten, waaronder gedragstudies die tot doel hebben om de functie van Sp4 te begrijpen, staan beschreven in *hoofdstuk 5*. Met behulp van gedragstudies kon het ontbreken van de paringsgedrag van de *Sp4*$^{-/-}$ mannetjes worden gelokaliseerd tot een defect in hun reukvermogen om niet-vluchtige geuren van bronstige vrouwtjes te herkennen. Daarnaast hadden de *Sp4*$^{-/-}$ mannetjes in het algemeen minder interesse in zowel vluchtige als niet-vluchtige geuren ten opzichte van wildtype mannetjes. In tegenstelling tot eerdere beschreven waarnemingen, vonden we dat ook de *Sp4*$^{-/-}$ vrouwtjes een sterk verminderd paringsgedrag vertoonden. Echter bij de vrouwtjes vindt het sterk verminderd paringsgedrag niet zijn oorsprong in een verstoorde capaciteit om onderscheid te maken tussen geuren van mannetjes of bronstige vrouwtjes. Verder wees bestudering van de gonaden van volwassen *Sp4*$^{-/-}$ muizen uit dat (1) de uterus bij de vrouwtjes een sterk verminderde inductie geeft in respons tot oestrogenen, (2) de gonaden van de mannetjes op latere leeftijd sterk gereduceerd zijn in omvang en (3) dat de mannetjes op latere leeftijd een

overgewicht ontwikkelingen dat op het conto komt van wit vetweefsel. Deze laatste observaties geven aan dat de werking van de androgene en/of oestrogene hormonen mogelijk verstoord zijn in de *Sp4*-deficiënte muizen.

In hoofdstuk 6 worden de biologische functies die Sp3 en Sp4 vervullen gedurende ontwikkeling en handhaving van een lichaam van een gewervelde bediscussieerd aan de hand van de informatie over de twee transcriptiefactoren die in dit proefschrift is gepresenteerd. Tevens wordt er een visie gegeven op onderzoek dat in de toekomst gedaan kan worden om de biologische functies van Sp3 en Sp4 verder in kaart te brengen.

Curriculum vitae

Naam	Pieter Fokko van Loo
Geboren	4 januari 1978 te Hoogeveen.
1990-1996	V.W.O. aan het Greijdanus College te Zwolle.
1996-2000	Doctoraal opleiding Moleculaire Wetenschappen aan de Wageningen Universiteit te Wageningen. Afstudeeronderzoek via de afdeling Biochemie van de Wageningen Universiteit te Wageningen bij de afdeling Celbiologie aan de Erasmus Universiteit te Rotterdam onder de supervisie van dr. Robbert Rottier en dr. Sjaak Philipsen. *Onderwerp: Purification of His-tagged Gata2 protein, to raise an antibody against Gata2.* Stage bij de afdeling Celbiologie aan de Erasmus Universiteit te Rotterdam onder de supervisie van dr. Dubi Drabek. *Onderwerp: Cloning of CD46 and CD55 by homologous recombination into one BAC construct.*
2000-2004	Assistent in opleiding bij de afdeling Celbiologie aan de Erasmus Universiteit te Rotterdam onder de supervisie van dr. Sjaak Philipsen en Prof. dr. Frank G. Grosveld. *Onderwerp: Studies on biological functions of the transcription factors Sp3 and Sp4.*
oktober 2004	Post-doc in de werkgroep van dr. Rudi W. Hendriks bij de afdeling Immunologie aan het ErasmusMC te Rotterdam.

Nawoord

Het afronden van mijn proefschrift markeert de afronding van mijn AIO-periode. Een periode waarin ik met plezier gewerkt heb aan het doorgronden van de biologische functies van de transcriptiefactoren Sp3 en Sp4.

Ik bedank mijn promotor Frank Grosveld en mijn co-promotor Sjaak Philipsen voor de ondersteuning bij het slagen van dit avontuur. Ik bedank mijn labgenoten, want zonder jullie had ik niet zoveel plezier gehad in mijn werk!

Daarnaast hebben de samenwerkingen met de groep van Rudi Hendriks (Department of Immunology, Erasmus MC, Rotterdam, The Netherlands), met Elaine Dzierzak en Kam-Wing Ling (Department of Cell Biology, ErasmusMC, Rotterdam, The Netherlands), met de mensen van de afdeling Anatomie en Embryologie (Department of Anatomie and Embryology, LUMC, Leiden, The Netherlands), met Jan Vreeburg en Marja Ooms (Department of Reproduction and Development, Erasmus MC, Rotterdam, The Netherlands) en met Julie Bakker (Centre for Cellular and Molecular Neurobiology, University of Liege, Liege, Belgium) een belangrijke bijdrage geleverd aan het tot stand komen van dit proefschrift. Bedankt voor jullie enthousiaste inzet.

Pa en ma, bedankt dat u er altijd bent als mijn vader en moeder!

Lieve Femmy, nu hebben we weer tijd om andere dingen te doen. Ik bedank je voor je steun en ruimte die je me hebt gegeven zodat ik mijn proefschrift kon afronden! Ik houd van je!