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Developmental Expression of Transcription Factors

Studies on Fox Proteins FoxF1, FoxF2 and FoxE3, BHLH-PAS Proteins Arnt and Arnt2 and Novel Arnt-Interacting Protein Tacc3

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ACADEMIC DISSERTATION

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To Petri
To Tuomas, Taru and Tuuli

Nature is always the same, and yet its appearance is always changing. It is our business as artists to convey the thrill of nature´s permanence along with the elements and the appearances of all its changes.

- *Paul Cezanne (ca. 1900)*

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, referred to in the text by using Roman numerals:

I Mahlapuu M, Pelto-Huikko M, Aitola M, Enerbäck S and Carlsson P (1998): FREAC-1 contains a cell-type-specific transcriptional activation domain and is expressed in epithelial-mesenchymal interfaces. Developmental Biology 202:183-195.

II Aitola M, Carlsson P, Mahlapuu M, Enerbäck S and Pelto-Huikko M (2000): Forkhead transcription factor *FoxF2* is expressed in mesodermal tissues involved in epithelio-mesenchymal interactions. Developmental Dynamics 218:136-149.

III Blixt Å, Mahlapuu M, Aitola M, Pelto-Huikko M, Enerbäck S and Carlsson P (2000): A forkhead gene, *FoxE3*, is essential for lens epithelial proliferation and closure of the lens vesicle. Genes & Development 14:245-254.

IV Aitola M and Pelto-Huikko M. The expression of *Arnt* and *Arnt2* mRNA in developing murine tissues. Accepted for publication by Journal of Histochemistry and Cytochemistry.

V Sadek C, Jalaguier S, Feeney E, Aitola M, Damdimopoulos A, Pelto-Huikko M and Gustafsson J-Å (2000): Isolation and characterization of AINT: a novel ARNT interacting protein expressed during murine embryonic development. Mechanisms of Development 97:13-26.

VI Aitola M, Sadek C, Gustafsson J-Å and Pelto-Huikko M. Aint/Tacc3 is highly expressed in proliferating mouse tissues during development, spermatogenesis and oogenesis. Submitted for publication.

ABBREVIATIONS

AD	activation domain
AFX	ALL1 fused gene from X chromosome
AhR	aryl hydrocarbon receptor
AhRR	AhR repressor
ALL	acute lymphoid leukemia
ARF	activin-responsive factor
Arnt	aryl hydrocarbon receptor nuclear translocator
Arnt2	aryl hydrocarbon receptor nuclear translocator 2
AZU-1	anti-zuai-1
bHLH	basic helix-loop-helix
bp	base pair
Bf	brain factor
Bmal	brain-muscle-Arnt-like
cAMP	cyclic adenosine monophosphate
CC10	Clara cell 10-kDa protein
cDNA	complementary deoxyribonucleic acid
CBP	CREB-binding protein
Cdk	cyclin-dependent kinase
ch-TOG	colonic and hepatic tumour overexpressed protein
Ci	cubitus interruptus
CPEB	cytoplasmic polyadenylation element binding protein
CREB	cAMP response element-binding protein
Cry	cryptochrome blue-light photoreceptor
CYP	cytochrome P450
dATP	deoxyadenosinetriphosphate
DBD	DNA binding domain
DFX	desferoxamine mesylate
Dhh	desert hedgehod
DNA	deoxyribonucleic acid
DP	dipyridol
dyl	dysgenetic lens
E-	embryonic day
ECTACC	endothelial cell-related TACC
eIF-4E	cap-binding translation initiation factor
EPAS1	endothelial PAS domain protein 1
ERIC-1	erythropoietin-induced cDNA-1
HSP	heat shock protein
FAST	forkhead activin signal transducer
FKH	forkhead homolog
FKHL	forkhead-like
FKHR	forkhead in rhabdomyosarcoma
FKHRL	FKHR-like
Fox	forkhead box
GAL	β-galactose

GAS41/NuBI1	glioma amplified sequence 41 / nuclear mitotic apparatus protein binding protein 1
Gli	glioma-associated oncogene
GFP	green fluorescent protein
GST	glutathione S-transferase
HFH	HNF-3/forkhead homolog
Hif	hypoxia inducible factor
HLF	Hif-1α like factor
HNF3α	hepatocyte nuclear factor
HRE	hypoxia-response element
IGFI	insulin-like growth factor I
Ihh	indian hedgehog
IR	immunoreactivity
MADS	DNA-binding domain named after the transcription factors Mcm1, Ag, DEFA and SRF
MAPK	mitogen-activated protein kinase
Mf	mesoderm/mesenchyme forkhead
Mfh	mesenchyme forkhead
MNF	myocyte nuclear factor
MOP	member of the PAS superfamily
mRNA	messenger ribonucleic acid
Msps	mini-spindles
NLS	nuclear localization signal
P-	postnatal day
PBS	phosphate-buffered salin
Pcna	proliferating cell nuclear antigen
PI3K	phosphatidylinositol-3 kinase
PAS	Per-Arnt-Sim
PAX	paired box
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PER	period
PKB	protein kinase B
Prox1	homeodomain protein related to *Drosophila prospero*
RNA	ribonucleic acid
Rpd3	reduced phosphate deficiency histone deacetylase
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulfate
Shh	sonic hedgehog
SIM	single minded
SPB	pulmonary surfactant protein B
TACC	transforming acidic coiled coil-containing protein
TAF	TATA-binding protein associated factor
TBP	TATA-binding protein
TFIIA,B,D,E,F,H	basal transcription factors associated with RNA polymerase II
TGF-β	transforming growth factor-β

Tim	timeless
Titf	thyroid transcription factor
TCDD	tetrachlorodibenzo-*p*-dioxin
twhh	tiggywinkle hedgehog
Whn	winged helix *nude*
XRE	xenobiotic response element

INTRODUCTION

To construct an embryo from a single cell is not an easy task. An embryo has to be able to respire before having lungs, digest before having a gut, form bones when still being mashy and create a network of neurons without knowing how to think. For a fertilized egg, it is possible to form a multicellular, functional organism, as it contains the inherited genes that transmit the developmental program from generation to generation. Genes guide the complex process of development, during which cells divide to enable growth, differentiate to specify separate organs and orientate themselves to organize three-dimensional structures. Nevertheless every single cell has got the same information, as the chromosomes were transmitted to the daughter cell. One of the central questions of developmental biology is to solve how the same genetic information leads cells to differentiate into numerous different cell types.

Research in molecular biology has shown that all the genes are not active at the same time. Instead they express at separate times to encode the formation of specific proteins needed by cells to be able to form an embryo, to continue development postnatally and to maintain the structural and functional integrity of adult tissues.

Selective gene expression is controlled by the specific transcriptional regulators. The initiation of transcription needs basal transcription factors which are common to all eukaryotic organisms, whereas specific transcription factors are proteins that bind DNA at specific sequences, sometimes far from the basal transcription machinery, and thereby influence transcription by either activating or repressing it. Each gene has a unique set of sites for binding a few of the thousands of specific transcription factors.

The activation of the transcription factors is the last step in a cascade leading to selective gene expression. Preceedingly, there are a variety of signal transduction pathways that originate from extracellular signals. These signals can be, for example, secreted molecules generated from inductive interactions with neighboring cells, hormones transported by the circulatory system or various stresses like mechanical or oxygen tension. In order to gain a nuclear response, these extracellular signals must be transmitted across the cell membrane and through the cytoplasm to reach the transcription factor targets. These factors may either be located in the nucleus or require subsequent translocation into the nucleus. The signaling pathways are extremely complex with intermediary proteins and multiple points of regulation. The commonest mechanism to transduce signals is reversible phosphorylation.

The number of known molecules involved in developmental regulatory processes is large. For example, 5 – 10 % of the genes in eukaryotic genomes encode regulatory factors that are dedicated to controlling the rate of transcription. While the structure and characteristics of these molecules have been revealed, less is known about where and when they exert their effects.

In this study, we present the temporal and spatial expression of certain proteins that posses, or are suggested to have, a role in the regulation of development. The results make it possible to evaluate the role of these proteins *in vivo* during development.

The importance of knowing the biological mechanisms regulating the expression of developmental genes is emphasized by the recent discoveries that mutations in these genes cause many diseases including congenital malformations and, unexpectedly, cancers. The progress in developmental biology has given us tools to elaborate prevention and treatment of many serious illnesses.

REVIEW OF THE LITERATURE

1. REGULATION OF GENE EXPRESSION DURING DEVELOPMENT

DNA (deoxyribonucleic acid) is the key molecule of life, as the inherited, genetic information needed for development and metabolism is encoded in the DNA sequences of the chromosomes. In eukaryotes (include animals, plants, fungi), the chromosomes are located to the nucleus, whereas prokaryotes (include bacteria) are devoid of nuclei. The information in the DNA determines the structures of proteins and contains thereby the instructions on how to develop from a fertilized egg into pluripotent stem cells and further along particular cell lineages into tissues and organs.

In a multicellular organism, every cell contains the same genetic information. However, not all the genes are transcribed at the same time or at the same level. To the contrary, every cell type expresses a different setup of genes which fulfill the specific needs of the cell. The gene expression can be regulated at the transcriptional level (which genes are transcribed into RNA), on RNA processing level (which of the transcribed RNAs are transported into the cytoplasm to become messenger RNAs), at the translational level (which of the mRNAs are translated into protein) and at the protein modification level (which proteins are allowed to stay functional in the cell) (Gilbert 1997).

1.1 RNA polymerase II transcription machinery

The transcription is regulated by a complex assembly of basal transcription initiation factors, sequence-specific DNA-binding transcriptional activators and repressors and associated cofactors on promoters, enhancers and silencers (Tjian and Maniatis 1994, St-Arnaud 1998). Next, transcriptional regulation is considered at the level of the basal transcription factors.

In eukaryotes, the basal transcription initiation apparatus includes RNA polymerase II and about 40 different subunits, at least TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (TF stands for transcription factor) (for review, see Orphanides et al. 1996, Mannervik et al. 1999). They are needed for accurate initiation of basal transcription *in vitro* and are directed to the 5´end of a transcription unit by the core promoter. *In vivo*, there is a sophisticated network of interactions between multiple upstream activators and the general transcription machinery.

The core of the promoter includes DNA elements recognized by general transcription machinery and is the assembly site of transcription machinery. Most core promoters include TATA-box, which is recognized by TATA-binding protein (TBP), a subunit of multimeric protein TFIID (Hernandez 1993). A strong TATA-box is usually connected with a high transcription rate of the gene and TATA-less promoters are often associated with a low transcription level of the gene (for review, see Pugh 2001). TFIID also contains TATA-binding protein-associated factors (TAFs) that have a role in determining TFIID to remain on promoter and as co-activators, forming bridges between enhancer-bound proteins and the transcription complex. TFIIA facilitates and stabilizes the binding of TFIID. TFIIB is needed for RNA polymerase II binding. TFIIF has enzymatic activity to unwind the DNA

helix and TFIIE is the energy source for transcription. The function of TFIIH is to release the RNA polymerase from the promoter region (for review, see Zawel and Reinberg 1995, Gilbert 1997).

There are a few ways in which gene expression can be regulated via the basal transcription machinery by activator proteins (for review, see Orphanides et al. 1996, Moreland et al. 1998, Kuras and Struhl 1999, Pugh 2000). In general, genes that are not transcribed, do not have the assembly of transcription complexes at their promoters. Before the core promoter DNA of a repressed gene is accessible, transcriptional activators are needed to unwrap the DNA from the nucleosomes. Specific transcriptional activators bind either to the naked DNA or to the chromatin templates, and assemble into a nucleoprotein complex termed enhanceosome. Then the enhanceosome recruits the RNA polymerase II holoenzyme (Thanos and Maniatis 1995, Wolffe and Pruss 1996, Carey 1998, Ellwood et al., 1999). Activator proteins are also needed to compete with the repressors from promoter DNA, direct the general transcription machinery to the proper promoter, induce conformational changes and covalent modification of proteins in the preinitiation complex, and stimulate promoter clearance and elongation.

1.2 Regulatory transcription factors

Where the basal transcription initiation apparatus is needed for initiation of transcription but is unable to regulate the rate of it, this task belongs to regulatory transcription factors. They communicate with the basal factors through coactivator proteins. The number of basal factors is limited, whereas the number of specific transcription factors is high. The expression of regulatory transcription factors may be limited to specific cell types or to a certain stage of development.

The regulatory transcription factors bind to *cis*-acting binding sites, located adjacent to the promoter, far upstream of it or even downstream of the gene. DNA looping allows regulatory proteins function even over long distances. Variations in the arrangements of the binding sites create unique conditions for the transcription factors to bind and form different nucleoprotein complexes individually for the needs of developmental genes.

A typical transcription factor has a modular structure containing a DNA-binding domain that directly contacts DNA and one or more transcriptional activation domains involved in interactions with coactivators and general transcription factors. As these domains may be incorporated in a variety of ways, even more regulatory specificity is obtained (reviewed by Tjian and Maniatis 1994).

A sequence-specific regulator needs a shape that allows it to form a significant surface area of contact with its response element in the DNA. Its surface chemistry should allow it to interact favorably with the charged phosphate backbone of the DNA as well as make sequence-specific bonds with bases. In order to increase the specificity of the protein-DNA interaction, many regulator proteins use multiple recognition modules that recognize additional features of DNA. They can also form homo- or heterodimers, so specifying a longer DNA sequence, or employ multiple DNA-binding domains by using repeats of the

same type of DNA-binding motif or by linking together different types of motifs (reviewed by Fairall and Schwabe 2001).

Sequence-specific regulators can be subdivided into activators or repressors, but several factors can mediate either activation or repression of the core promoter utilization in a context-dependent manner. The changes in concentration of a transcription factor may affect whether it acts as an activator or as a repressor, or the response may be dependent upon the ligands present, or the physiological state of the cell (reviewed by Courey 2001).

The expression of regulatory transcription factors needs to be regulated itself (Gilbert 1997). Their synthesis can be regulated by other transcription factors. Often their enhancers and promoters are very complex, allowing them to be expressed only in certain cells (Blackwood and Kadonaga 1998). In some cases, phosphorylation is needed to activate the dormant regulator, but it can also be used to repress transcription factors (Hunter and Karin 1992). The formation of heterodimers between *trans*-acting proteins can alter their ability to activate transcription, their affinity for DNA or sequence specificity.

1.3 Cofactors

The enhancement of transcription needs additional mediator proteins and coactivators, in addition to the transcriptional activator proteins (Goodrich et al. 1996). They can selectively potentiate the stimulatory activity of specific subsets of enhancer binding transcriptional activators (reviewed by St-Arnaud 1998). Coactivators link together the basal transcription factors and regulatory transcription factors and help in recruiting or activating the preinitiation complex but they do not bind directly to the DNA. Coactivators can also catalyze covalent or non-covalent changes in chromatin structure altering the accessibility of the template to the general transcription machinery (reviewed by Courey 2001).

The number of multi-subunit coactivator complexes discovered is increasing, but the number of coactivators is not as high as that of the specific DNA-binding factors. Among the best-known cofactors are the TBP-associated factors (TAFs), which are components of the general transcription machinery and function as coactivators mediating interactions between the general transcription machinery and regulatory transcription factors and activate transcription. They may have a role in core promoter recognition and promoter topology changes (Tansey and Herr 1997). TAFs can also function as an enzyme. They can have histone acetyltransferase activity and thus be involved in DNA accessibility to activators and general factors or they can phosphorylate themselves and the basal factor TFIIF.

In addition to coactivators, also corepressors are needed to mediate communication between diverse upstream regulatory proteins and the core RNA polymerase II transcription complex. Corepressors inhibit the binding or function of RNA polymerase II transcription complex. For example reduced phosphate deficiency histone deacetylase (Rpd3) has been identified as a critical co-repressor of various mammalian transcription factors (reviewed by Mannervik et al. 1999). It is thought to condense chromatin at the core promoter or enhancer, thus preventing the interaction between activators and transcription initiation machinery.

1.4 Transcription factor families

Transcription factors can be divided into several families based on the structure utilized for DNA binding or sometimes on the activation domain or oligomerization domain (Mitchell and Tjian 1989, Tjian and Maniatis 1994). The members of the same transcription family usually have highly homologous DNA-binding surfaces, but different surface residues outside the DNA-binding region. These various residues are important in determining the specific roles for each member of the family. The members of the same family often share also other structural or functional properties like the ability to form heterodimers or the mechanism to transmit extracellular signals. There are several different DNA-binding domains and motifs that classify transcription families, for example, homeodomain and Myb domain, classical zinc finger motif, MADS domain, basic-region-leucine-zipper domain, forkhead motif and bHLH (basic helix-loop-helix) motif (reviewed by Pabo and Sauer 1992 and Fairall and Schwabe 2001).

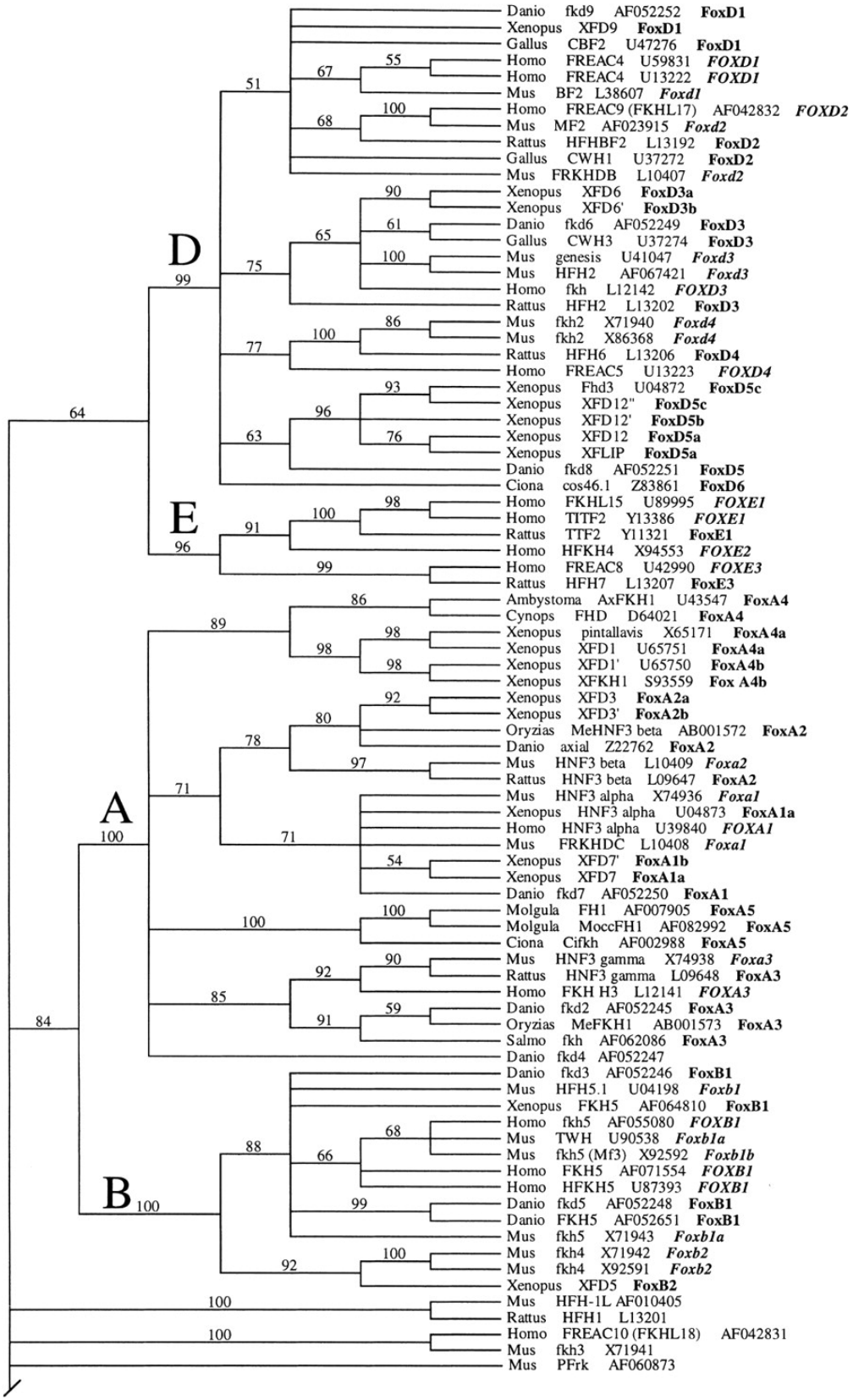
2. FORKHEAD FAMILY OF TRANSCRIPTION FACTORS

2.1 General aspects of forkhead proteins

The forkhead family of transcription factors is characterized by a highly conserved DNA binding motif, a forkhead motif (Weigel and Jäckle 1990). The first gene of this rapidly growing family was the *fork head* (*fkh*) gene in *Drosophila* (Weigel et al. 1989). The *fkh* mutations cause homeotic transformations of the ectodermal portion of the gut causing replacement of foregut and hindgut by ectopic head structures in embryos. This phenomenon led to the name "fork head". Soon, the fork head motif was found also in rat hepatocyte specific factors HNF3α, -β and –γ (Lai et al. 1990, Lai et al. 1991). Since then, over 100 genes encoding members of the forkhead family have been identified in a variety of eukaryotes ranging from invertebrates to humans (reviewed by Kaufman and Knöchel 1996).

2.1.1 Nomenclature

The confusions caused by multiple names and classification systems of a rapidly growing forkhead gene family were solved in 2000 by a committee that revised the nomenclature to reflect the phylogenetic relationships between the family members more accurately (Kaestner et al. 2000). The symbol *Fox,* for *Forkhead box*, was adopted as the unified symbol for all vertebrate genes encoding forkhead transcription factors. Fifteen subclasses have been delineated for known chordate Fox proteins. The constantly updated phylogenetic tree can be downloaded from the web at http://www.biology.pomona.edu/fox.html (Fig.1). Fox protein subclasses were designated by a capital letter and within each subclass proteins were given an Arabic numeral. Abbreviations for the chordate Fox proteins will contain all capital letters for human (e.g., FOXF1), only the first letter capitalized for the mouse (e.g., Foxf1) and the first and subclass letters capitalized for all other chordates (e.g., FoxF1). Italicized *versus* roman letters are used to distinguish genes and proteins, respectively.


Danio fkd9 AF052252 FoxD1
Xenopus XFD9 FoxD1
Gallus CBF2 U47276 FoxD1
Homo FREAC4 U59831 FOXD1
Homo FREAC4 U13222 FOXD1
Mus BF2 L38607 Foxd1
Homo FREAC9 (FKHL17) AF042832 FOXD2
Mus MF2 AF023915 Foxd2
Rattus HFHBF2 L13192 FoxD2
Gallus CWH1 U37272 FoxD2
Mus FRKHDB L10407 Foxd2
Xenopus XFD6 FoxD3a
Xenopus XFD6' FoxD3b
Danio fkd6 AF052249 FoxD3
Gallus CWH3 U37274 FoxD3
Mus genesis U41047 Foxd3
Mus HFH2 AF067421 Foxd3
Homo fkh L12142 FOXD3
Rattus HFH2 L13202 FoxD3
Mus fkh2 X71940 Foxd4
Mus fkh2 X86368 Foxd4
Rattus HFH6 L13206 FoxD4
Homo FREAC5 U13223 FOXD4
Xenopus Fhd3 U04872 FoxD5c
Xenopus XFD12" FoxD5c
Xenopus XFD12' FoxD5b
Xenopus XFD12 FoxD5a
Xenopus XFLIP FoxD5a
Danio fkd8 AF052251 FoxD5
Ciona cos46.1 Z83861 FoxD6
Homo FKHL15 U89995 FOXE1
Homo TITF2 Y13386 FOXE1
Rattus TTF2 Y11321 FoxE1
Homo HFKH4 X94553 FOXE2
Homo FREAC8 U42990 FOXE3
Rattus HFH7 L13207 FoxE3
Ambystoma AxFKH1 U43547 FoxA4
Cynops FHD D64021 FoxA4
Xenopus pintallavis X65171 FoxA4a
Xenopus XFD1 U65751 FoxA4a
Xenopus XFD1' U65750 FoxA4b
Xenopus XFKH1 S93559 Fox A4b
Xenopus XFD3 FoxA2a
Xenopus XFD3' FoxA2b
Oryzias MeHNF3 beta AB001572 FoxA2
Danio axial Z22762 FoxA2
Mus HNF3 beta L10409 Foxa2
Rattus HNF3 beta L09647 FoxA2
Mus HNF3 alpha X74936 Foxa1
Xenopus HNF3 alpha U04873 FoxA1a
Homo HNF3 alpha U39840 FOXA1
Mus FRKHDC L10408 Foxa1
Xenopus XFD7' FoxA1b
Xenopus XFD7 FoxA1a
Danio fkd7 AF052250 FoxA1
Molgula FH1 AF007905 FoxA5
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Ciona Cifkh AF002988 FoxA5
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Rattus HNF3 gamma L09648 FoxA3
Homo FKH H3 L12141 FOXA3
Danio fkd2 AF052245 FoxA3
Oryzias MeFKH1 AB001573 FoxA3
Salmo fkh AF062086 FoxA3
Danio fkd4 AF052247
Danio fkd3 AF052246 FoxB1
Mus HFH5.1 U04198 Foxb1
Xenopus FKH5 AF064810 FoxB1
Homo fkh5 AF055080 FOXB1
Mus TWH U90538 Foxb1a
Mus fkh5 (Mf3) X92592 Foxb1b
Homo FKH5 AF071554 FOXB1
Homo HFKH5 U87393 FOXB1
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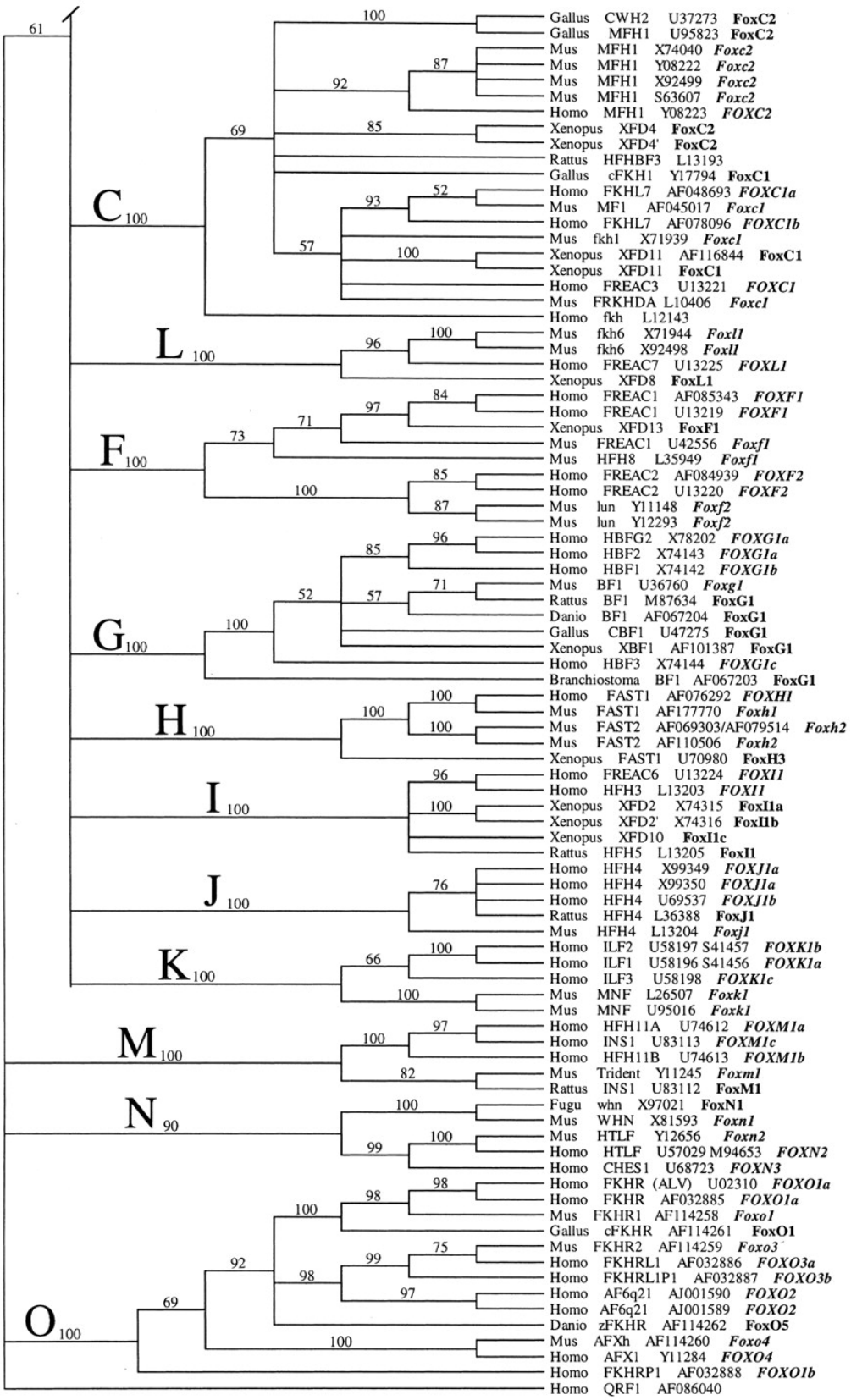

FIGURE 1. Phylogenetic tree of chordate Fox proteins.

2.1.2 Structure of the DNA-binding domain

The 100 amino acid DNA binding site of FoxA3 (HNF3γ) bound to DNA has been crystallized and the three-dimensional structure determined by Clark and co-workers (1993). The forkhead domain of FoxA3 (HNF-3γ) is a variant of the helix-turn-helix motif. It includes two loops or wings on the C-terminal side of the helix-turn-helix and this butterfly shaped structure is also called the "winged helix motif". The forkhead motif binds DNA as a monomer and binding of some forkhead transcription factors to their cognate sites has been shown to cause alternations in DNA topology by bending it at an angle of 80-90˚ (Pierrou et al. 1994).

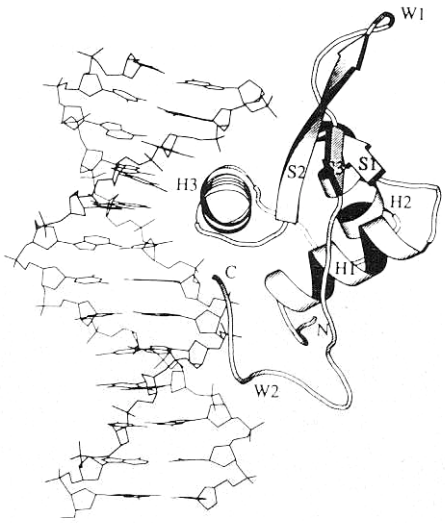

FIGURE 2. The three dimensional structure of the DNA-binding domain of FoxA3 bound to DNA. The α-helices of the N-terminal part are labeled as H1, H2 and H3 and a three-stranded, twisted, antiparallel β-sheet forming β-strands as S1, S2 and S3. α-helix H3 is lying in the major groove of DNA making the direct base contacts. The loop W1 connects strands S2 and S3 and interacts with DNA. The domain is stabilized by the loop W2 that appears between S3 and C-terminus. (Modified from Clark et al. 1993.)

2.1.3 DNA-binding specificity

In several cases, the DNA-binding domains of distantly related Fox proteins are surprisingly similar, and because of the structural resemblance of the DNA-binding domains, the target DNA sites are sometimes overlapping or identical (Pierrou et al. 1994, Kaufmann et al. 1995, for review, see Kaufmann and Knöchel 1996). A common 7 bp recognition core motif 5′ [(G/A) (T/C) (C/A) A A (C/T) A] 3′ has been revealed from binding sites of several different forkhead factors among several separate species (for review, see Kaufmann and Knöchel 1996). This core motif is necessary but not sufficient for transcription factor recognition. The flanking sequences and the terminal residues of the core motif are needed for binding specificity (Pierrou et al. 1994, Overdier et al. 1994, Kaufmann et al. 1995, Roux et al. 1995).

FOXD1 and FOXD2 have identical amino acid sequences of the forkhead motif although they have no similarities outside of this DNA binding motif and are encoded by genes located on separate chromosomes (Ernstsson et al. 1997). *FOXD1* and *FOXD2* transcripts have overlapping expression patterns in the kidney and COS-7 and 293 cell lines (Ernstsson et al. 1996, Ernstsson et al. 1997). Their relative abundance may be a crucial determinant for their function as transcriptional regulators. In case of overlapping or identical target DNA sites, more specificity for transcription factors may be provided by a divergent temporal and spatial expression pattern or context dependence. Other domains except DNA binding domain, like trans-activation or repression domains, are highly divergent among Fox proteins.

2.1.4 Signal transduction pathways

Most of the Fox proteins are transcriptional activators, but some are transcriptional repressors (Sutton et al. 1996, Freyaldenhoven et al. 1997, Bourguignon et al. 1998), whereas some may have both roles depending on the context (Tan et al. 1998). Fox proteins have been shown to participate in the signal transduction pathways of the TGF-β superfamily. Members of the TGF-β superfamily of signaling molecules function by activating transmembrane receptors with phosphorylating activity; these in turn phosphorylate and activate proteins of the Smad family, a class of signal transducers (Kretzschmar and Massague 1998). TGF-β superfamily is critical in the establishment of mesoderm during early embryogenesis in *Xenopus* (Hemmati-Brivanlou and Melton 1992, Dale et al. 1993, Jones et al. 1995, Kessler and Melton 1995).

FoxH3 has the ability to mediate transcriptional induction by activin in *Xenopus* embryos (Chen et al. 1996, Chen et al. 1997, Watanabe and Whitman 1999, Yeo et al. 1999) (Fig. 3). It has a role as a transcriptional partner for Smad proteins as it associates with Smad2 and Smad4 to form a transcriptionally active complex, ARF (activin-responsive factor). This multiprotein complex binds on the promoter of the *Xenopus mix.2* gene in response to stimulation by several TGF-β superfamily ligands. ARF binds to an enhancer through both FoxH3 and Smad binding sites but only DNA binding by FoxH3 is necessary for ARF binding or transcriptional regulation by activin (Yeo et al. 1999). In the absence of activin signaling, FoxH3 does not activate transcription of the *mix.2* gene (Harland and Gerhart 1997). The human and mouse homologs of FoxH3 have also been shown to have a role in TGF-β signaling pathways (Labbe et al. 1998, Weisberg et al. 1998, Zhou et al. 1998, Liu et al. 1999).

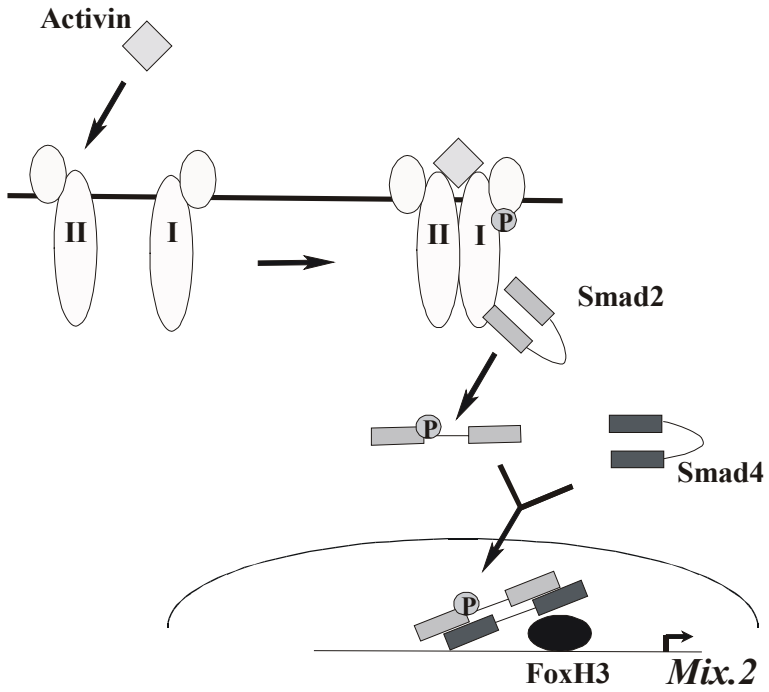

FIGURE 3. Model for activin signaling through transmembrane receptor kinases, Smad proteins and FoxH3. Activin binds to type II cell-surface serine/threonine kinase receptor and recruits and phosphorylates type I receptor. Activated type I receptor then interacts and phosphorylates Smad2 enabling it to interact with Smad4. The heteromeric complex translocates to the nucleus and is recruited by FoxH3 to the promoter of *Mix.2* to activate transcription in early *Xenopus* embryo. (Modified from Attisano and Wrana 2000, Wrana and Attisano 2000)

The winged helix transcription factors LIN-31 and LIN-1 Ets have been shown to mediate MAPK (mitogen-activated protein kinase) signaling specificity during *Caenorhabditis elegans* vulval induction (Tan et al. 1998). LIN-31 and LIN-1 Ets are direct targets of MAPK and both can be phosphorylated by MAPK. The MAPK phosphorylation of LIN-31 prevents the binding of unphosphorylated LIN-31 and LIN-1 Ets to each other, thus relieving vulval inhibition. Phosphorylated LIN-31 may also promote vulval cell differentiation by acting as a transcriptional activator (Tan et al. 1998).

Another example of signal transduction pathways where forkhead transcription factors are substrates to the protein kinase cascades is the one where the subfamily of forkhead transcription factors is a target of protein kinase B (PKB, also known as c-akt) (reviewed by Kops and Burgering 1999 and Kops and Burgering 2000). PKB has become known as a proto-oncogene (Bellacosa et al. 1991, Staal 1987). In mammalian cells, PKB is activated by growth factors, certain cytokines and cellular stress (reviewed by Coffer et al. 1998). In the presence of a ligand, the pathway is initiated at receptor tyrosine kinases. The next step is the activation of the heterodimeric phosphatidylinositol-3 kinase (PI3K). Active PI3K produces 3´phosphorylated inositol lipids that act as second messengers to recruit PKB to the plasma membrane and thus PKB is activated and released into the cytosol (Franke et al. 1997). Activated PKB is capable of phosphorylating several proteins and is thereby involved in several cellular processes like apoptic signaling, protein translation, nitric oxide production and metabolic processes (reviewed by Kops and Burgering 2000). PKB can mediate effects on transcription via mammalian transcription factors FOXO4, FOXO1 and

FOXO3 (Brunet et al. 1999, Guo et al. 1999, Kops et al. 1999, Rena et al. 1999, Takaishi et al. 1999, Tang et al. 1999) as well as *C. elegans* DAF-16 (Ogg et al. 1997, Paradis and Ruvkun 1998, Paradis et al. 1999, Cahill et al. 2001) (Fig. 4).

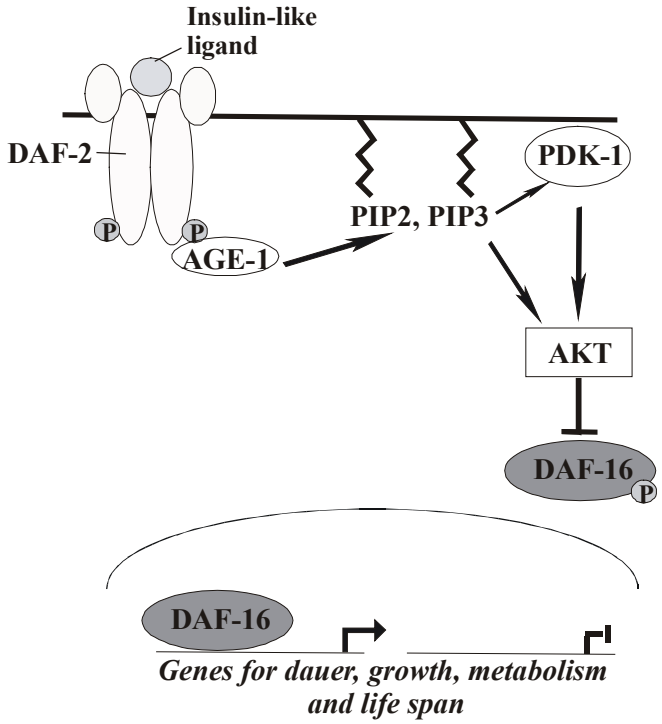

FIGURE 4. Model for insulin-mediated inhibition of DAF-16-dependent transcription in *C. elegans.* An insulin-like ligand binds to and activates DAF-2, an insulin/insulin like growth factor – like receptor. This recruits and activates the AGE-1/P13K following the generation of phosphoinositides PIP2 and PIP3 that are required for PDK-1 and AKT-1/AKT-2 (homologs of mammalian PKB) activation. Activated AKT kinases are able to move to the nucleus and phosphorylate forkhead transcription factor DAF-16 resulting in inhibition of target gene expression. Unphosphorylated DAF-16 could activate genes necessary for dauer arrest, metabolism and increased life span or could repress genes necessary for reproductive growth. Modified from Kops and Burgering 1999 and Paradis et al. 1999.

FOXO1, FOXO3 and FOXO4 have been shown to contain 3 PKB phosphorylation sites and to be phosphorylated and regulated following addition of insulin or IGFI to cells (Brunet et al. 1999, Guo et al. 1999, Kops et al. 1999, Rena et al. 1999). The phosphorylation leads to the inactivation of these forkhead transcription factors by promoting export from the nucleus (Biggs et al. 1999, Brunet et al. 1999). This may affect cellular survival, metabolism and cell cycle progression (Kops and Burgering 1999). For FOXO4, a Ras signaling pathway has also been identified (Kops et al. 1999, Medema et al. 2000).

Several mammalian Fox genes have been shown to be involved in the Hedgehog family signaling pathways. The members of the Hedgehog family of signaling molecules play a crucial role during invertebrate and vertebrate development as they mediate patterning processes and they also have an important role in the generation of several cancers (reviewed by Hammerschmidt et al. 1997, Ingham 1998). The *Hedgehog* gene was first discovered in *Drosophila*, whereas several vertebrate *hedgehog* homologues have also been

cloned, including *Sonic hedgehog (Shh), Indian hedgehog (Ihh), Desert Hedgehog (Dhh)* and *tiggywinkle hedgehog (twhh)*. Most Hedgehog signaling leads to the activation of transcriptional effectors of the Ci/Gli family (reviewed by McMahon 2000).

In certain developing tissues, Shh has been shown to induce the expression of some forkhead transcription factors. For example, *Foxc2* and *Foxd2* expression in the presomitic mesoderm is dependent on Sonic hedgehog signals from the notochord in inducing the formation of the vertebral column (Furumoto et al. 1999, Wu et al. 1998). In the floorplate of the neural tube, Shh induces the expression of *Foxa2* (Roelink et al. 1995).

2.1.5 Developmental role

A critical role of the Fox transcription factors for both vertebrate and invertebrate development has been shown in a number of studies where Fox genes have been inactivated by gene targeting or mutations. The results for studies performed on the mouse are shown in table 1.

TABLE 1. The phenotypes of mice resulting from inactivation of *Fox* gene by gene targeting or by spontaneous mutation

Gene	Phenotype	References
Foxa1 (HNF-3α)	Mutant mice die postnatally, decreased circulating glucagon levels	Kaestner et al. 1999, Shih et al. 1999
Foxa2 (HNF-3β)	Mutant embryos die at E8.5, absence of organized primitive node and notochord formation	Ang and Rossant 1994, Weinstein et al. 1994, Sund et al. 2000
Foxa3 (HNF-3γ)	Mutant mice develop normally, are fertile, no morphological defects but levels of expression of several target genes are reduced	Kaestner et al. 1998
Foxb1 (Mf3, Fkh5, HFH-e5.1)	Mutant mice display variable phenotypes, e.g. perinatal mortality, growth retardation, nursing defects and central nervous system abnormality	Labosky et al. 1997, Wehr et al. 1997, Alvarez-Bolado et al. 2000
Foxc1 (Mf1)	Mutant mice die at birth with multiple defects, e.g. hydrocephalus, ocular, renal, urinary tract, skeletal and cardiovascular abnormalities	Kume et al. 1998, Kidson et al. 1999, Winnier et al. 1999, Kume et al. 2000a, Smith et al. 2000
Foxc2 (Mfh1)	Mutant embryos die perinatally and display skeletal and cardiovascular defects	Iida et al. 1997, Winnier et al. 1997, Winnier et al. 1999, Kume et al. 2000a, Smith et al. 2000
Foxd1 (Bf2)	Homozygotes die within 24 hours after birth with abnormal kidneys	Hatini et al. 1996
Foxd2 (Mf2)	Mutant mice are viable and fertile but 40% have renal abnormalities	Kume et al. 2000b
Foxe1 (Titf2)	Homozygotes die within 48 hours after birth and exhibit cleft palate and either a sublingual or completely absent thyroid gland	De Felice et al. 1998
Foxe3	Mutations cosegregate with *dysgenetic lens (dyl)* phenotype	Blixt et al. 2000 (Paper III), Brownell et al. 2000
Foxf1 (Freac1, HFH-8)	Mutant embryos die before E10 due to defects in mesodermal differentiation and cell adhesion	Mahlapuu et al. 2001a
Foxg1 (Bf1)	Mutant embryos die at birth and have a dramatic reduction in the size of the cerebral hemispheres	Xuan et al. 1995
Foxh1 (FAST)	Mutant embryos die prenatally, fail to pattern the anterior primitive streak and form primitive node,	Hoodless et al. 2001

	prechordal mesoderm, notochord, and definitive endoderm	
Foxi1 (Fkh-10)	Mutants exhibit malformations of the inner ear resulting in deafness and disturbed balance	Hulander et al. 1998
Foxj1 (HFH-4)	Most mutant mice die before weaning and display defects in ciliogenesis and left-right axis formation	Chen et al. 1998, Brody et al. 2000
Foxk1 (MNF)	Mutant mice are viable but exhibit growth retardation and atrophic skeletal muscles with impaired regeneration ability	Garry et al. 2000
Foxl1 (Fkh-6)	Most mutant mice die before weaning and have dysregulation of epithelial cell proliferation in intestines	Kaestner et al. 1997
Foxm1 (Trident, HFH-11)	Mutant mice die perinatally and have nuclear abnormalities in cardiomyocytes and hepatocytes	Korver et al. 1998
Foxn1 (Whn)	Athymic nude mouse	Nehls et al. 1994, Nehls et al. 1996
Foxp3	The defective gene is found in the *scurfy* mouse mutant which is characterized by over-proliferation of CD4+CD8- T lymphocytes, extensive multiorgan infiltration and elevation of numerous cytokines	Brunkow et al. 2001
Foxq1 (HFH1L)	The defective gene is found in Satin homozygous mice that have a silky coat with high sheen arising from structurally abnormal medulla cells and defects in differentiation of the hair shaft	Hong et al. 2001

The phenotypes of mammalian models where *Fox* genes have been inactivated, show similarities with human congenital disorders. So far, studies have been able to indicate a few Fox genes to be associated with human hereditary diseases. The results of relevant studies are shown in table 2.

TABLE 2. The Fox genes associated with human hereditary disorders

Gene	Phenotype	References
FOXC1 (FREAC3, FKHL7)	Defects of the anterior segment of the eye associated with developmental forms of glaucoma	Lehmann et al. 2000, Mears et al. 1998, Mirzayans et al. 2000, Nishimura et al. 1998, Nishimura et al. 2001
FOXC2 (Mfh1)	Lymphedema-distichiasis (lymphedema of the limbs, double rows of eyelashes, may also include cardiac defects, cleft palate, extradural cysts, photophobia	Fang et al. 2000
FOXE1 (TITF-2)	Thyroid agenesis, cleft palate, choanal atresia	Clifton-Bligh et al. 1998
FOXE3 (FREAC8)	Anterior segment ocular dysgenesis and cataract	Semina et al. 2001
FOXL2	Blepharophimosis/ ptosis/epicanthus syndrome (BPES) type I (associated with ovarian failure) and II	Crisponi et al. 2001, Prueitt and Zinn 2001
FOXN1 (Whn)	T-cell immunodeficiency combined with lack of hair and dystrophic nails	Frank et al. 1999
FOXP2	Disorders of speech and language	Lai et al. 2001
FOXP3	Immune dysregulation, polyendocrinopathy, enteropathy syndrome (IPEX)	Bennett et al. 2001, Wildin et al. 2001

2.1.6 Role in tumorigenesis

Disruption of genes involved in developmental processes will, in many cases, lead to cancer, indicating that cancer ensues from errors in the developmental program. A subset of Fox transcription factors has been shown to be involved in tumorigenesis. The first oncogene that was reported to encode a forkhead protein was *FoxG1,* a retroviral oncogene from the Avian sarcoma virus 31 (Li and Vogt 1993). *FoxG1* displays a particular homology to the mammalian forkhead gene *Foxg1,* which is necessary for the development of the cerebral hemispheres of mice (Tao and Lai 1992, Xuan et al. 1995). FoxG1 is a transcriptional repressor and induces oncogenic transformation by down-regulating the expression of specific target genes (Li et al. 1997, Xia et al. 2000).

Chromosomal translocations that cause leukemia or rhabdomyosarcoma are associated with genes for FOXO2, FOXO4 and FOXO1. The translocations result in the creation of highly active chimeric transcription factors, MLL -FOXO2, MLL - FOXO4 and PAX3 - FOXO1 (Sublett et al. 1995, Borkhardt et al. 1997, Hillion et al. 1997, Anderson et al. 2001). Since FOXO4 is a X-linked gene, a translocation in males results in loss of FOXO4 activity. In

addition to the active, oncogenic chimeric protein formation by translocation, the loss of FOXO4 activity can cause tumorigenesis by downregulating the inhibition of cell growth (Kops and Burgering 2000). Respectively, PAX3 - FOXO1 fusion protein is a more potent activator than the wild type PAX3 and can act as an oncogenic transcription factor by enhancing activation of normal PAX3 target genes (Fredericks et al. 1995). On the other hand, this fusion protein can upregulate the genes encoding MET receptor and platelet-derived growth factor α (PDGF-α) receptor, which are strong activators of PKB and thereby inactivators of FOXO1 (Epstein et al. 1998, Ginsberg et al. 1998).

2.2 Characteristics of forkhead transcription factors FOXF1, FOXF2 and FOXE3

Here is described what was known about FOXF1/Foxf1, FOXF2/Foxf2 and FOXE3 before our results in Paper I, II and III were published. The later information is reviewed in the discussion section of this thesis.

2.2.1 FOXF1

Human *FOXF1* was cloned from a fetal human cDNA library by Pierrou and coworkers (1994) and it was previously called *FREAC1*. It was shown to be expressed in the adult and fetal lung and placenta by northern blot (Pierrou et al. 1994). The chromosomal localization in humans was found to be 16q24 (Larsson et al. 1995). Hellqvist and co-workers (1996) published the full-length cDNA sequences for *FOXF1* cloned from the human lung cDNA library. Cotransfections with a reporter carrying FOXF1 binding sites showed that FOXF1 is a transcriptional activator and the activation domain was found in the C-terminal side of the forkhead domain (Hellqvist et al. 1996). In search for genes regulated by FOXF1, human *SPB* (pulmonary surfactant protein B) and *CC10* (Clara cell 10-kDa protein) promoters were found to be activated (Hellqvist et al. 1996).

A mouse cDNA sequence very similar to *FOXF1* was reported by Clevidence and co-workers (1994) and it was called *HFH-8*. However the predicted amino acid sequence was very different from that of FOXF1, which was later shown to be due to the deletions and insertions that caused frameshifts (Hellqvist et al. 1996). Clevidence and the co-workers (1994) reported the expression of *FoxF1* in type II pneumocytes in adult rat lung. The same laboratory evaluated later the expression of *Foxf1* in developing and adult (Peterson et al. 1997, Kalinichenko et al. 2001). These results are reviewed later in discussion part.

2.2.2 FOXF2

The sequence for human *FOXF2* was published in the same paper as that of *FOXF1* (Pierrou et al. 1994). It was previously called *FREAC2/FKHL6*. The mouse homologue has been described also under the name *LUN* (Miura et al. 1998). In the preliminary work, the northern blot analysis revealed almost identical patterns of expression for *FOXF1* and *FOXF2* (Pierrou et al. 1994). In addition to the fetal and adult lung, expression was observed at low levels in the prostate, small intestine, colon and fetal brain (Pierrou et al. 1994). The mouse *Foxf2* mRNA has been reported to express in adult lung and in small intestine by Northern blot analysis (Miura et al. 1998). The expression in the adult lung was localized to bronchiolar epithelial cells and type II pneumocytes by *in situ* hybridization analysis (Miura et al. 1998).

FOXF1 and FOXF2 have been shown to have many structural and functional similarities although they are encoded by distinct genes as *FOXF2* gene was localized to chromosomal position 6p25.3 (Blixt et al. 1998). FOXF2 is a transcriptional activator and contains two activation domains in the C-terminal part of the protein (Hellqvist et al. 1996, Hellqvist et al. 1998) (Fig. 5).

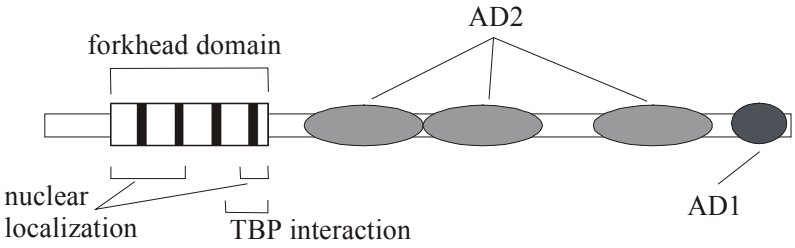

FIGURE 5. Schematic view of the location of DNA-binding domain (forkhead domain) and two independent activation domains (AD1 and AD2) in FOXF2. AD1 consists 23 amino acids in the C-terminal end of the protein and shares homology to the C terminus of FOXF1. AD2 is built up by three synergetic subdomains and is spread out over approximately 200 amino acids in the central part of the protein. Modified from Hellqvist et al. 1998.

FOXF2 binds to and activates the surfactant protein B promoter in a similar manner to FOXF1. Interestingly, *CC10* is activated only by FOXF1 due to the presence of a cell type-specific activation domain (Hellqvist et al. 1996). The DNA-binding forkhead domain of human FOXF2, mouse Foxf2 and mouse Foxf1 are 100% identical while that of human FOXF1 differs by 3 amino acid substitutions (Clevidence et al. 1994, Pierrou et al. 1994, Hellqvist et al. 1996) (Fig. 6). Outside the DNA-binding domain, the C-terminal activation domain is the only region where FOXF2 and FOXF1 have similarities (Pierrou et al. 1994, Hellqvist et al. 1998).

FIGURE 6. Clustal alignment of amino acid sequences of human FOXF2, mouse Foxf2, human FOXF1 and mouse Foxf1 (Thompson et al. 1994). Identical amino acids are bolded and the forkhead DNA-binding domains are highlighted with grey. FOXF2, Foxf2 and Foxf1 are identical within the forkhead domain and there is only three amino acids difference with the forkhead domain of FOXF1.

FOXF2 has been shown to interact with the general transcription factors TBP and TFII *in vitro* (Hellqvist et al. 1998). TBP interaction site on FOXF2 is located on the C-terminal end of the forkhead domain but the purpose of this interaction is unclear (Hellqvist et al. 1998) (Fig. 5). Transcriptional activation was enhanced by overexpression of TFIIB in cotransfection experiments and this was dependent on the C-terminal part of the protein containing the activation domains. This finding suggests that the transcriptional activity of FOXF2 is mediated at least partly through recruitment of TFIIB (Hellqvist et al. 1998).

Evidence for nuclear localization of FOXF2 comes from the presence of nuclear localization signals (NLSs) amino acid motifs that cause a protein to be translocated to the nucleus through docking with NLS receptors, within the DNA binding domain of FOXF2 (Hellqvist et al. 1998) (Fig 5).

2.2.3 FOXE3

A partial nucleotide sequence of human *FOXE3* cDNA was reported 1995 as a result of screen for human forkhead genes (Larsson et al. 1995). The chromosomal localization was mapped to1p32 (Larsson et al. 1995).

3. bHLH-PAS FAMILY OF TRANSCRIPTION FACTORS

3.1 General aspects of bHLH-PAS proteins

The basic-helix-loop-helix-PAS (bHLH-PAS) proteins are transcriptional regulators which have been isolated from several species and control a variety of developmental and physiological events including xenobiotic metabolism, hypoxic response, circadian rhythms and neurogenesis (reviewed by Rowlands and Gustafsson 1997 and Crews 1998). They usually function as dimeric DNA-binding protein complexes. The most common unit is a heterodimer between family members although some members can form homodimers (Antonsson et al. 1995, Sogawa et al. 1995). The number of known non-related interacting partners is increasing (Whitelaw et al. 1993, Gekakis et al. 1995, Arany et al. 1996, Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer et al. 1998). Usually another partner is broadly expressed whereas the expression of another is spatially or temporally restricted, or dependent on the presence of inducers (reviewed by Crews 1998). As bHLH-PAS proteins have interactive and competitive properties, they are capable of regulating transcription via a variety of mechanisms. They are widely expressed among animals, plants and prokaryotes and function as signal transducers between the environment and the transcriptional machinery of cells, although some other still unknown mechanisms for these proteins may exist. The research on bHLH-PAS proteins has provided insight into their multiple roles.

The superfamily of bHLH-PAS proteins can be divided into subgroups according to functional similarities (sensors, partners, and coactivators) or evolutionary relatedness (α, β and γ class) (reviewed by Gu et al. 2000). The functions can be overlapping, since many members of this superfamily can act either as sensors of an environmental signal or as general dimerization partners. The sensors include e.g. aryl hydrocarbon receptor (AhR) and hypoxia-inducible factors Hif-1α, Hif-2α and Hif-3α (Burbach et al. 1992, Wang and Semenza 1995, Hogenesch et al. 1997). Aryl hydrocarbon receptor nuclear translocators Arnt, Arnt2 and Arnt3 serve as partners which direct several sensor bHLH-PAS proteins to their cognate enhancer elements (Swanson et al. 1995, Hirose et al. 1996, Ikeda and Nomura 1997, Takahata et al. 1998, Michaud et al. 2000). The γ-class coactivators form a special subgroup within the bHLH-PAS proteins, as they have not been shown to dimerize with other bHLH-PAS proteins but are involved in transcriptional activation of steroid receptors (Glass et al. 1997).

3.1.1 bHLH- and PAS motifs

The sequence organization of bHLH-PAS proteins is highly conserved (Fig. 7). The bHLH (Fig. 8) domain is located near the amino terminus. The basic region forms an α-helix that interacts with the major groove of DNA (Ma et al. 1994). The HLH motif derives its name from a region of conserved amino acids that gives rise to a secondary structure of two amphipathic α-helices separated by a relatively unconserved loop (Kadesch 1992). The HLH motif serves as a dimerization domain (Murre et al. 1989, Kadesch 1993). The

transcriptional activation or repression domains are situated in the carboxy-terminal residues (Franks and Crews 1994, Jain et al. 1994, Li et al. 1994, Moffett et al. 1997). PAS is an acronym formed from the names of the first proteins identified with this motif: the *Drosophila* Period (Per), vertebrate aryl hydrocarbon receptor nuclear translocator (Arnt), and the *Drosophila* Single-minded (Sim) (Nambu et al. 1991). The PAS domain typically encompasses 250-300 amino acids and contains a pair of highly conserved 50 amino acid subdomains termed the A and B repeats (Hoffman et al. 1991, Jackson et al. 1986, Nambu et al. 1991). The PAS domain is found immediately carboxy-terminal to the bHLH domain. It can mediate a variety of biological functions like dimerization between PAS proteins (Huang et al. 1993), small molecule binding (Dolwick et al. 1993a, Coumailleau et al. 1995) and interaction with non-PAS proteins (Coumailleau et al. 1995, Gekakis et al. 1995, Arany et al. 1996, Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer et al. 1998).

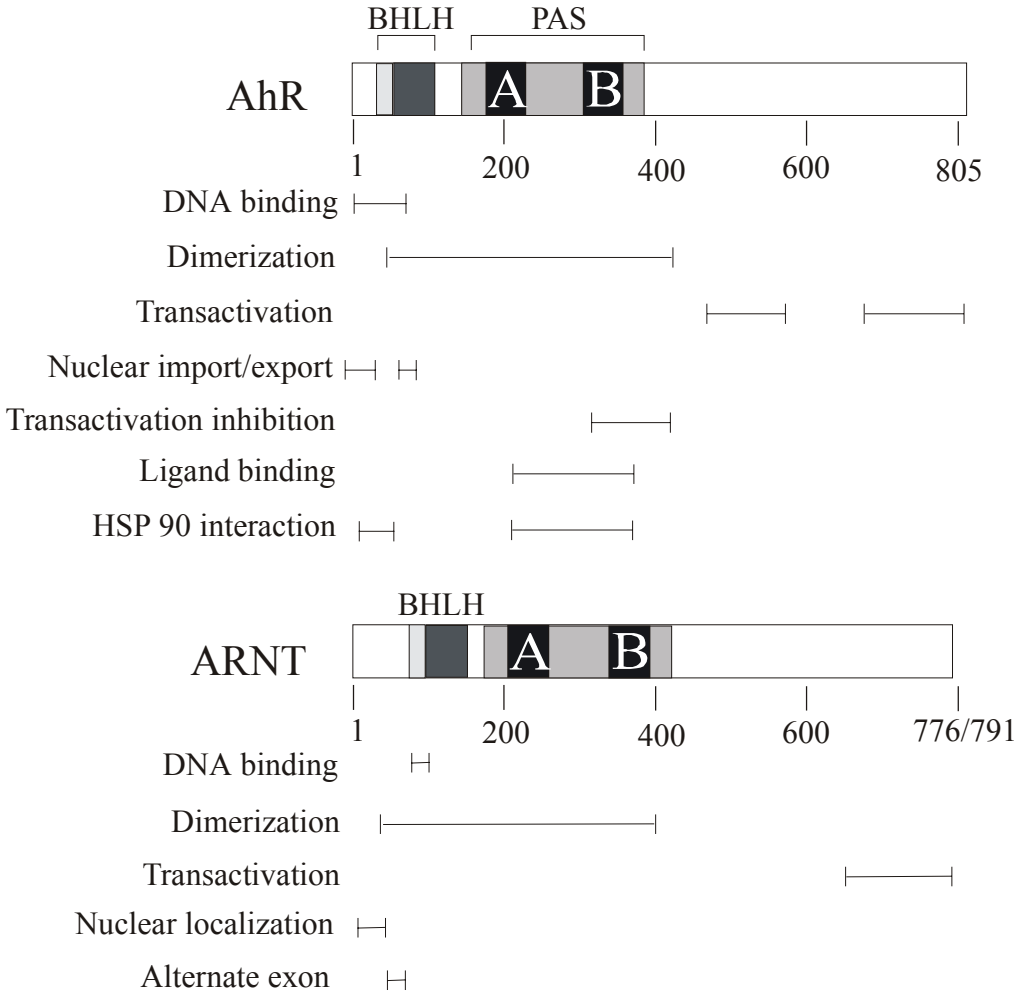

FIGURE 7. Schematic representation of bHLH/PAS family members AhR and Arnt. Two hydrophobic repeat sequences, denoted A and B, within the PAS domain, are indicated by solid boxes. (Figure adapted from Gu et al. 2000.)

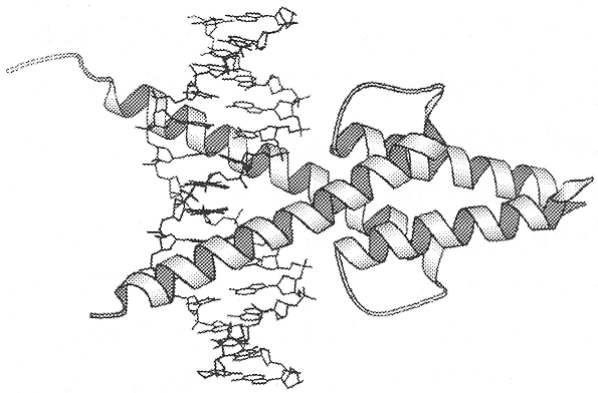

FIGURE 8. The structure of the homodimer of the bHLH DNA-binding domain of MyoD complexed with DNA (modified from Ma et al. 1994).

3.2 bHLH-PAS dependent response pathways

Expanding research on bHLH-PAS proteins has provided models of signal transduction pathways that represent the ideas about how these protein heterodimers regulate transcription as a response to different environmental signals.

3.2.1 Aryl hydrocarbon receptor pathway

The aryl hydrocarbon receptor pathway allows animals to adapt to an environment contaminated with planar aromatic compounds by upregulating batteries of xenobiotic metabolizing enzymes and thus shortening the biological half-life of the harmful chemicals (reviewed by Rowlands and Gustafsson 1997, Crews 1998, Gonzalez and Fernandez-Salguero 1998, Gu et al. 2000). Studies have also suggested a role for this pathway in cell cycle regulation and apoptosis (reviewed by Nebert et al. 2000).

The aryl hydrocarbon receptor, AhR, is among the most extensively studied bHLH-PAS proteins and its function has been described in great detail, although some models of mechanisms are contradictory. The human, mouse, and rat AhRs have been cloned (Burbach et al. 1992, Dolwick et al. 1993b, Carver et al. 1994). AhR heterodimerizes with another bHLH-PAS protein Arnt in order to form a functional complex. This complex is induced by lipophilic ligands, e.g. dioxin, which bind to AhR. To date, no endogenous ligand for AhR has been identified, although several dietary molecules have been reported to be able to bind to this receptor, inducing transcription (Ciolino et al. 1998). Report of AhR knockout mice showed that an endogenous ligand for AhR probably exists, as the constitutive expression of cytochrome P-450 1A2 is absent in these animals (Fernandez-Salguero et al 1995).

In the absence of a ligand, AhR exists in a latent conformation in a complex with a dimer of HSP90 and additional cellular chaperones such as ARA9 (also called AIP1 or XAP2) and p23 (Ma and Whitlock 1997, Carver et al. 1998, Meyer et al. 1998, Kazlauskas et al. 1999). The HSP90 is required both for maintaining the AhR in a latent non-DNA binding state and a ligand-binding conformation (Pongratz et al. 1992, Whitelaw et al. 1995).

The noxious environmental compounds that bind to AhR include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls, and polycyclic aromatic

hydrocarbons (for a review, see Rowlands and Gustafsson 1997). They cause acute and chronic toxicity and may be carcinogens. Upon ligand binding, the HSP90 molecules are displaced from the AhR, which then enters the nucleus and dimerizes with Arnt. This heterodimer binds to an E-box motif in xenobiotic response elements to increase the rate of transcription of specific target genes (Fig. 9). These include genes for the xenobiotic-metabolizing enzymes *CYP1A1*, *CYP1B1, CYP1A2*, the glutathione S transferase Ya subunit and quinone oxidoreductase (reviewed by Whitlock 1999, Taylor and Zhulin 1999). Transcriptional activation has been suggested to be mediated by the co-activator CBP/p300, which connects AhR-Arnt heterodimer with the TATA box-associated factors (Kobayashi et al. 1997). This results in recruitment of RNA polymerase II. Also protein kinase C-dependent phosphorylation has been suggested to participate in gene activation by AhR (Chen and Tukey 1996).

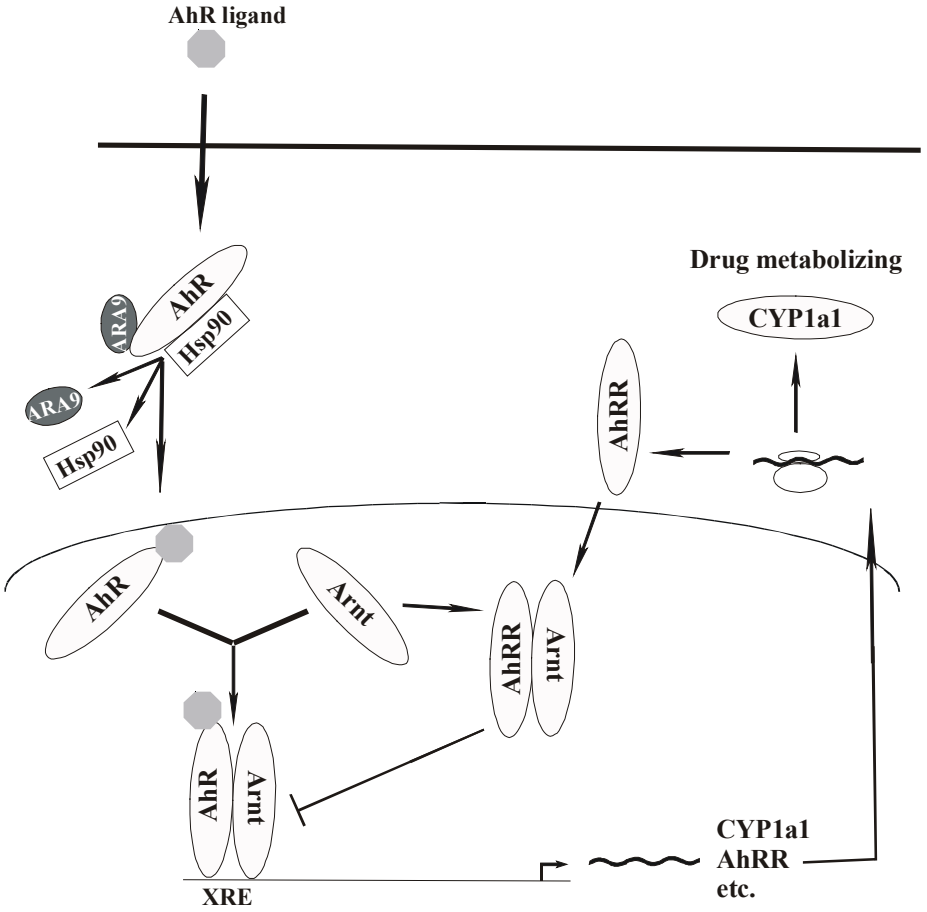

FIGURE 9. Model of the aryl hydrocarbon receptor pathway. Ahr acts as a cytoplasmic receptor that is maintained in a ligand-responsive state as a complex with Hsp90 and ARA9. On activation by its ligand, Ahr translocates from cytoplasm into the nucleus and exchanges its chaperones with ARNT. The Ahr-ARNT heterodimer binds to xenobiotic responsive element (XRE) and activates transcription of downstream target genes including the *AhRR* gene. The resulting AhRR inhibits Ahr function by competing with AhR for heterodimerizing with Arnt and binding to the XRE. (Modified from Crews and Fan 1999, Mimura et al. 1999, Whitlock 1999 and Gu et al. 2000.)

In addition to the induction of xenobiotic-metabolizing enzymes, the ligand-bound AhR has been found to activate gene expression of the AhR repressor (AhRR) (Mimura et al. 1999). AhR and AhRR compete for dimerizing with Arnt and binding to the XRE sequence thus providing a feedback inhibition mechanism. It has been suggested that the Arnt homologue Arnt2 takes part in the aryl hydrocarbon receptor pathway as an alternate partner for the AhR (Hirose et al. 1996, Drutel et al. 1996).

3.2.2 Hypoxia response pathway

The hypoxia response pathway allows organisms to adapt to changes in atmospheric and cellular oxygen. Hypoxia can arise e.g. during embryogenesis, wound healing and tumor growth. Hypoxia-inducible factor-1α plays an important role in O_2 homeostasis (reviewed by Wenger and Gassmann 1997 and Semenza 1998). Under normoxic conditions, Hif-1α is degraded through the ubiquitin-proteasome pathway. As a response to hypoxia, Hif-1α is translocated from cytoplasm to nucleus and binds to hypoxia response elements (HREs) of target genes, like those encoding erythropoietin, vascular endothelial growth factor (VEGF), glucose transporters and glycolytic enzymes, as a heterodimeric complex with Arnt (Hif-1β) (Levy et al. 1995, Wang et al. 1995, Wang and Semenza 1995, Jiang et al. 1996, Iyer et al. 1998). The transcriptional activity is potentiated by the general activator CBP/p300 (Arany et al. 1996, Kallio et al. 1998). As a result of transcriptional activation, the organism can adapt to hypoxia via stimulated erythropoiesis, increased vascular bed density, vascular permeability and glycolysis (reviewed by Bunn and Poyton 1996 and Gu et al. 2000).

In addition to Hif-1α and Arnt also other members of bHLH/PAS family have been found to play a role in hypoxic pathways. These proteins include Hif-2α (also referred to as EPAS1/HLF/MOP2) and Hif-3α, which dimerize with Arnt in response to reduced oxygen tension (Ema et al. 1997, Tian et al. 1997, Flamme et al. 1997, Gu et al. 1998). Arnt2 and Arnt3 (MOP3/Bmal), homologs of Arnt, may serve as β-class partner of the α-class Hif sensor subunits (Drutel et al. 1996, Hirose et al. 1996, Hogenesch et al. 1997, Ikeda and Nomura 1997, Takahata et al. 1998).

3.2.3 Circadian response pathway

The circadian response pathway adapts an animal's activity to its illuminated environment. To maintain circadian rhythms, an organism needs both an internal clock and the ability to respond to environmental cues that keep the clock in tune (reviewed by Dunlap 1999 and Gu et al. 2000). In mammals, the circadian pacemaker is located in the suprachiasmatic nucleus (SCN) and the light signal that resets the pacemaker is received by the photoreceptor of the retina and transmitted to the SCN through the retinohypothalamic tract (Inouye and Kawamura 1979, Klein and Moore 1979).

A group of bHLH-PAS proteins controls circadian rhythms. Clock and MOP3 (Arnt3/ Bmal1) are expressed in the suprachiasmatic nucleus and retina of rodents and show a circadian rhythm responding to light (King et al. 1997, Abe et al. 1999, Honma et al. 1998, Namihira et al. 1999). They form a heterodimer that binds to the response element (M34RE, the MOP3 and MOP4 responsive element or a circadian responsive E-box) and positively regulates circadian rhythm-expressed genes like *Per* (Gekakis et al. 1998). The levels of Per

proteins and their localization into the nucleus oscillate in a circadian manner (Zwiebel et al. 1991, Curtin et al. 1995). The circadian response pathway also involves a number of non-PAS proteins that interact with bHLH-PAS proteins. These non-PAS proteins include Tim, which is thought to translocate to the nucleus with Per, and the cryptochrome genes Cry1 and Cry2 which have been shown to interact with Per and Tim (Sehgal et al. 1995, Saez and Young 1996, Ceriani et al. 1999, Kume et al.1999). Per, Tim and Cry are negative components in the mammalian clock feedback loop (Darlington et al. 1998, Kume et al.1999).

3.3 bHLH-PAS proteins AhR, Arnt, Arnt2 and Hif-1α as regulators of mammalian development

bHLH-PAS proteins control a variety of developmental events. They are expressed widely during development, and dysregulated expression of many bHLH-PAS proteins is associated with developmental abnormalities and tumorigenesis. Recent studies, which have been made using mouse knockouts of bHLH-PAS genes have provided new information about their roles in mammalian development.

3.3.1 AhR

The wide expression of *AhR* during mouse embryonic development suggests a developmental role for this protein. The *Ahr* expression has been detected in the pituitary, palatal shelf, nasal septal cartilage, tongue, thymus, lung, liver, gut, kidney, bladder, urogenital sinus, tip of genital tubercle and in the inner layer of the retina in the mouse (Jain et al. 1998) and also in the neuroepithelium, branchial arches, somites, heart, adrenal, bone and muscle (Abbott et al. 1995). In the developing embryo, the AhR-ligand complex can mediate many biological responses like toxicity, induction of a cleft palate and hydronephrosis (Peterson et al. 1993). In many adult mammalians the *AhR* is generally expressed in the liver, kidney, lung, heart, thymus, brain and placenta (Dolwick et al. 1993b, Carver et al. 1994, Kainu et al. 1995).

The reports of the *AhR* null mice shed further light on the role of AhR in development. The $AhR^{-/-}$ mice created by Fernandez-Salguero et al. (1995) have demonstrated that AhR plays a critical role in the development of the liver and immune system. Almost half of the $AhR^{-/-}$ mice died soon after birth. Those that died had lymphocyte infiltration of various organs including the gut, urinary tract and lung. The survivors grew more slowly than their normal littermates but were fertile. The livers of $AhR^{-/-}$ mice were reduced in size and these animals developed fibrosis in the portal area and cholangitis. The accumulation of lymphocytes was decreased in the spleen and lymph nodes of $AhR^{-/-}$ mice. *AhR* null allele mice have also been created by Schmidt et al. (1996) and Mimura et al. (1997). The mice created by Schmidt et al. were viable and fertile though they grew slowly and also had liver pathology. No apparent pathology was detected in the phenotype of a third independently generated *AhR* null mice (Mimura et al. 1997). AhR knock-out mice have been shown to fail in up-regulating batteries of xenobiotic metabolizing enzymes in response to exposure to dioxin and to have an altered teratogenic response (Mimura et al. 1997, Peters et al. 1999).

3.3.2 Arnt

Arnt, the common heterodimerization partner in the bHLH-PAS family, has been shown to be widely expressed in the developing mouse (Abbott and Probst 1995, Jain et al. 1998). This expression has been observed during embryonic and postnatal mouse development in the neuroepithelium of the brain and spinal cord, branchial arches, heart, liver, gut, lung, tongue, palate, pharynx, bone, muscle, adrenal gland, submandibular gland, testes, brown fat, thymus, kidney, bladder and otic and optic placodes (Abbott and Probst 1995, Jain et al. 1998).

Targeted mutation of the murine *Arnt* locus causes embryonic mortality between E9.5 and E10.5 (Kozak et al. 1997, Maltepe et al. 1997). The $Arnt^{-/-}$ embryos have abnormalities such as neural tube defects, forebrain hypoplasia, delayed rotation of the embryo, placental hemorrhaging, and visceral arch abnormalities.

3.3.3 Arnt2

The embryonic expression of the aryl hydrocarbon receptor nuclear translocator 2 (Arnt2), the close relative of Arnt, has been thought to be mainly restricted to the neural tissues (Hirose et al. 1996, Jain et al. 1998). Homozygous *Arnt2* gene knockout mouse embryos die perinatally and exhibit impaired hypothalamic development similar to those of bHLH-PAS gene *Sim1* (Single-minded-1) knockout mice suggesting that Sim1 and Arnt2 presumably interact *in vivo* (Michaud et al. 2000, Hosoya et al. 2001, Keith et al. 2001). *Arnt2* and *Arnt* have a strong genetic interaction shown by crossed compound heterozygous mutant $Arnt^{+/-}$, $Arnt2^{+/-}$ mice embryos (Keith et al. 2001). The embryos with fewer than two wild-type alleles of either *Arnt* or *Arnt2* were under-represented suggesting overlapping functions for *Arnt* and *Arnt2* during embryonic development.

3.3.4 Hif-1α

Hif-1α has been shown to express widely during mouse embryonic development (Iyer et al. 1998, Jain et al. 1998). The tissues expressing *Hif-1α* during mouse embryogenesis include the myocardial wall, atrioventricular canal, neuroepithelium, branchial arches, liver, gut, thymus, kidney and olfactory epithelium (Jain et al. 1998). The role of Hif-1α as a master regulator of O_2 homeostasis during embryogenesis is emphasized by the observations of the $Hif\text{-}1a^{-/-}$ knockout mice (Iyer et al. 1998, Ryan et al. 1998, Kotch et al. 1999, Semenza et al. 1999). The $Hif\text{-}1a^{-/-}$ embryos died by E11 and had neural tube defects, cardiovascular malformations and massive cell death throughout the embryo. The Hif-1α- as well as Arnt-deficient stem cells were lacking the normal induction of VEGF, glucose transporters and glycolytic enzymes mRNA expression induced by hypoxia (Maltepe et al. 1997, Iyer et al. 1998, Ryan et al. 1998). $Hif\text{-}1a^{-/-}$ stem cells also had a reduced capacity to form teratocarcinomas compared to wild-type stem cells when injected into immunocompromised mice (Ryan et al., 1998). This was correlated with reduction in tumor vascular density.

4. TACC PROTEIN FAMILY

4.1 Representation of the TACC family

The members of the ***t***ransforming, ***a***cidic, ***c***oiled-***c***oil-containing protein family share a conserved, C-terminal coiled coil domain. The exact physiological roles of these proteins

are not known. TACC proteins are suggested to be involved in mitotic spindle assembly and they have been shown to be capable of forming multiple different protein complexes in cell (Gergely et al. 2000a, Gergely et al. 2000b, Lee et al. 2001, Lauffart et al. 2002). There is evidence for the association of TACC proteins with both developmental and oncological processes (Still et al. 1999a, Still et al. 1999b, Chen et al. 2000).

The first human gene identified was *TACC1,* which was characterized from human breast cancer amplification, 8p11 (Still et al. 1999a). Other published human TACC proteins are TACC2/AZU1, TACC3/ ERIC1 and ECTACC (Still et al. 1999a, Still et al. 1999b, Chen et al. 2000, Pu et al. 2001), which are encoded by genes associated with genomic regions rearranged in certain cancers. The mouse homologues Tacc2 (accession no. NM021314) and Tacc3/Eric1 (accession no. AF093543/ AF247674), have been cloned (Still et al. 1999b). Additional members of this family have been found in rabbit (TACC4, the sequence of which has been submitted to GenBank, accession no. AF372837), *Drosophila* (D-TACC) and *Xenopus* (Maskin) (Stebbins-Boaz et al. 1999, Gergely et al. 2000a).

The C-terminal coiled coil domain identifies the TACC protein family. The conserved 200 amino acid TACC domain is predicted to form a coiled-coil structure, which is a bundle of several helices (Lupas 1996). It is similar to the rod-like domains which are found in proteins like myosin (Still et al. 1999a). The coiled-coil domains of TACC1, TACC2, TACC3 and ECTACC contain a predicted tyrosine phosphorylation site within the C-terminus (Still et al. 1999a, Still et al. 1999b).

4.2 Characteristics of TACC family

4.2.1 TACC1

Transforming acidic coiled coil-containing gene 1, *TACC1*, has been shown to express in several adult human tissues including the heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. During early murine embryonic development, at E7, Northern blot analysis has revealed a high expression of *Tacc1* mRNA expression. This expression was down-regulated by E11 (Still et al. 1999a).

TACC1 encodes an 805 amino acids protein, which is rich in serine, proline and acidic residues (Still et al. 1999a). Within the C-terminal coiled coil domain there is a conserved tyrosine phosphorylation site, which may regulate coiled coil domain function. In addition to homology with the coiled coil domain of TACC2 and TACC3, similarities are found with coiled coil domains of yeast tropomysin and other nuclear proteins involved in mitotic spindle assembly. Two typical nuclear localization signals are located within the N-terminal serine/proline/acidic rich region (Still et al. 1999a).

TACC1 is expressed at high levels in human cancer cell lines (Still et al. 1999a). *In vitro* analysis of TACC1 has shown that constitutive expression results in transformation and anchorage independent growth of mouse fibroblasts suggesting that TACC1 might promote malignant growth (Still et al. 1999a).

TACC1 has been shown to be weakly concentrated in the centrosomes during mitosis and to interact with a microtubule-associated colonic and hepatic tumor overexpressed human ch-

TOG protein (Gergely et al. 2000b, Lauffart et al. 2002). Another known partner of TACC1 is an oncogenic transcription factor GAS41/NuBI1 (Lauffart et al. 2002).

4.2.2 TACC2/ECTACC/AZU1

The cDNA of *TACC2* has been mapped as part of the genome mapping project (Schuler et al. 1996). It was mapped to 10q26, another chromosome region amplified in breast cancer (Courjal et al. 1997). *TACC2* is highly related to *TACC1*. Anti-zuai-1 (AZU-1) is same as TACC2 as they are identical with the exception of two insertions and one amino acid substitution (Chen et al. 2000). Also the sequence of endothelial-TACC-related *ECTACC* cDNA is nearly identical to partial *TACC2* cDNA (Pu et al. 2001). AZU-1 has been suggested to be a tumor suppressor in breast tumor as it has been shown to be abundantly expressed in phenotypically normal and premalignant mammary epithelial cells but dramatically down-regulated in a variety of breast carcinoma cell lines and carcinomas in situ. Re-expression of AZU-1 in human tumorigenic mammary epithelial cells reduced the malignant phenotype of cells in culture and *in vivo* (Chen et al. 2000).

ECTACC was found in searching for erythropoietin–responsive genes in human microvascular endothelial cells by differential display (Pu et al. 2001). It was shown to be upregulated by erythropoietin treatment. The expression of *ECTACC* mRNA was seen in human heart and skeletal muscle (Pu et al. 2001).

4.2.3 AINT/TACC3/ ERIC1

The human *TACC3* has been cloned by Still and colleagues as a result of searching for TACC family members in the dbEST database (Still et al. 1999b). The murine *Tacc3* gene has also been constructed from EST sequences (Still et al. 1999b). The mouse Tacc3 contains 541 amino acids and human TACC3 837 amino acids. The coiled coil domain is 60% identical with the corresponding domain of TACC1. Human ERIC1 (McKeveney et al. 2001) and mouse Eric1 (accession no. AF247674) share a high degree of identity with TACC3 and are likely to be variants of the same protein.

TACC3 localizes to 4p16, a region disrupted in multiple myeloma (Still et al. 1999b). TACC3 has been shown to be upregulated in various cancer cell lines indicating a role in cell growth and differentiation. Northern blot analysis has detected its expression in the human testis, in lower levels in the thymus and at very low levels in other tissues of the immune system. Murine Tacc3 was expressed through embryonic stages from E7 to E17 with the highest expression at E15 (Still et al. 1999b). The subcellular localization of TACC3 has not yet been identified.

4.2.4 D-TACC

Drosophila transforming acidic coiled-coil (D-TACC) protein was biochemically isolated from the *Drosophila* embryo when proteins interacting with microtubules were sought (Gergely et al. 2000a). The antiserum against D-TACC stained centrosomes strongly and microtubules weakly throughout the cell cycle in fly embryos. It has been shown to interact with mini-spindles protein Msps and have a strong effect on microtubule behavior in *Drosophila* embryos (Lee et al. 2001). Msps could not accumulate around the centrosomes in an appropriate way when D-TACC levels were reduced, and this resulted in

destabilization of the centrosomal microtubules (Lee et al. 2001). Mutations in D-TACC cause defects in chromosomal segregation during mitosis in *Drosophila* embryos (Gergely et al. 2000a).

4.2.5 Maskin

A conserved C-terminal coiled-coil has also been found in the frog: *Xenopus* Maskin is 70% identical to human TACC3 (Stebbinz-Boaz et al. 1999). It interacts with CPEB (cytoplasmic polyadenylation element binding protein) and eIF-4E (cap-binding translation initiation factor) to restrict polyadenylation-induced translation during oocyte maturation (Stebbinz-Boaz et al. 1999). It has been shown to be maintained through the midblastula stage of *Xenopus* and to decline in the gastrula and neurula stages. It was also evident in testis but not in several somatic tissues studied (Stebbinz-Boaz et al. 1999).

AIMS OF THE PRESENT STUDY

The puzzle how an organism develops from a single fertilized oocyte to an integrated selection of differentiated cells that form tissues capable of communicating with each other and comprising an appropriately functioning unit, is under intense investigation by developmental biologists. A key to solve this problem is to study the regulation of gene expression during development. Novel proteins participating in the signaling network controlling gene expression are being found at an accelerating pace. Sometimes, little is known about the functions of these new proteins. In order to increase understanding of the regulation of gene expression during development, this study was initiated. We have searched and characterized new proteins involved in the developmental processes in the mouse and rat, studied possible interactions of these proteins, mapped the expression patterns during development and analyzed spontaneous mutants in order to find clues about their function.

Specific aims of the study were:

- to investigate and compare the developmental expression of genes encoding closely related forkhead transcription factors *Foxf1* and *Foxf2* mRNAs in development of the mouse/rat in order to find clues about the similarities and differences concerning their biological functions (I-II)
- to study the expression of Foxe3 in order to understand its biological role (III)
- to examine the expression of *Arnt* and *Arnt2* mRNA in the developing mouse (IV)
- to examine gene and protein expression of a novel Arnt-interacting protein in the developing and adult mouse (V, VI)

MATERIALS AND METHODS

1. EXPERIMENTAL ANIMALS

Embryonic (E9-E17), postnatal (1.5, 2, 3, 8 days and two weeks old) and adult NMRI mice, embryonic (E9-E20) Sprague-Dawley rats (University of Tampere) were used in this study for papers I, II, IV, V and VI. For paper III, Balb/c (Charles River) and *dyl/dyl* mice (Jackson Laboratory, Maine) were obtained.

2. LIBRARY SCREENING, CLONING AND DNA SEQUENCING (I, III, V)

The sequencing of *FOXF1/FREAC1* (I) was performed by subcloning relevant fragments into pBluescript SK from a human placenta cosmid library in the vector pWE15 (Evans et al. 1989) spanning the entire *FOXF1* locus (Larsson et al. 1995). The nucleotide sequences were determined on an Amersham Thermosequenase or a Pharmacia ALF sequencer using fluorescein-labelled primers.

Foxe3 (III) clones were isolated after screening a genomic 129/Sv λ library (Stratagene) with a probe from the previously cloned human *FOXE3* gene (Larsson et al. 1995). A fragment centered around the forkhead box was subcloned and sequenced on a Beckman CEQ2000 using GPS-1 transposon insertions (New England Biolabs, Beverly, MA).

For comparison of wild-type *Foxe3* sequence with respective sequence of *dyl/dyl* mouse genome, DNA was prepared from Balb/c (Charles River) and *dyl/dyl* mice. *Foxe3* was amplified in two overlapping pieces with primers (GGGATGGGGCCCAGAGACTGACTC and CGCAGGAAGCTACCGTTGTCGAAC; GCCCTACTCATACATCGCGCTCAT and TGGAGGAGGGCAGGGAAGGCTTAG).

In order to find novel Arnt interacting proteins, GAL4 MATCHMAKER yeast two-hybrid system was performed as suggested by the manufacturer (Clontech, Palo Alto, CA) (V). Arnt was fused to the DNA binding domain and fragments of 17-day mouse embryo cDNA library to the activation domain using recombinant vectors as described in paper V. The fusion proteins were expressed in *Saccharomyces cerevisiae* strains HF7C and Y187 and the strains were mated in order to find interacting proteins. Positive clones were selected on the basis of ability to grow on synthetic dropout plates lacking leucine, tryptophan, and histidine. Mated yeast went through growth and ß-galactosidase assays as described in paper V. As a positive control, the assay was performed using GADAhR or GADHif-1α. The Aint/Tacc3 clone obtained from the yeast two-hybrid screening was used as a probe to screen a 16-day mouse embryo cDNA library in a λExlox vector (Novagen, Madison, WI) as recommended by the manufacturer. Ten positive recombinant phage were isolated out of 3×10^5 phage and additional two rounds of screening were completed to isolate pure positive phage. Autosubcloning was carried out and insert size determined by polymerase chain reaction (PCR) as described in paper V. The sequencing was carried out using the ABI AmpliTaq sequencing kit (Perkin-Elmer, Wellesley, MA).

3. GENE CHARACTERIZATION (I, III)

In order to study the genomic organization of the human *FOXF1/FREAC1* gene, relevant fragments were subcloned in pBluescript SK(-) from a human placenta cosmid library in the vector pWE15 (Evans et al. 1989) spanning the entire *FOXF1* locus (Larsson et al. 1995) (I). The nucleotide sequences were determined on an Amersham Thermosequenase or a Pharmacia ALF sequencer using fluorescein-labelled primers.

In search of chromosomal localization of *Foxe3,* fluorescent *in situ* hybridization of mouse metaphase chromosomes was carried out with a 9-kb digoxigenin-labeled probe as previously described (Helou et al. 1998) (III). After hybridization, the signals were visualized with FITC-anti digoxigenin and banding patterns by DAPI counterstaining.

4. PROTEIN ANALYSIS

4.1. Transfections, luciferase and gel shift assays (I)

In order to investigate the transcriptional activation domains of FOXF1/FREAC1, deletions were made in the *FOXF1/FREAC1* cDNA with *Bal*31 nuclease to generate plasmids that express C-terminally truncated forms of FOXF1/FREAC1. Relevant segments of *FOXF1/FREAC1* cDNA were PCR amplified with primers, tagged with *Sal*I and *Xba*I sites and cloned into the corresponding sites of pNG4 in order to fuse parts of FOXF1/FREAC1 to the DNA binding domain of GAL4. Luciferase reporter plasmids containing the apoB minimal promoter and multiple binding sites for FOXF1/FREAC1 and GAL4, have been described elsewhere (Hellqvist et al. 1996, 1998).

Cell lines (human fetal lung cell line W138, the green monkey kidney cell line COS-7 and the human epithelial cell line HeLa) were grown according to the protocol described in paper I. Cells were cotransfected with expression constructs encoding different FOXF1/FREAC1 variants and a luciferase reporter containing multiple FOXF1/FREAC1 binding sites and assayed for luciferase activity (Promega, Technical Bulletin No. 101, Madison, WI) as previously described (Hellqvist et al. 1996, 1998). Gel shift assay with probes containing a FOXF1/FREAC1 (GATCCAACGTAAACAATCCGAGATC) or Gal4 (GATCCGGACTGTCCTCCGAGATC) DNA-binding site and nuclear extracts from cells transfected with the constructs was performed as described in paper I.

4.2 *In vitro* protein-protein interaction assays (V)

To determine which region of Aint/Tacc3 protein is required for interaction with Arnt, *in vitro* protein-protein interaction assays were performed. For that, Aint/Tacc3 was divided into three PCR-amplified sections, which were fused to the GAL4AD and yeast two-hybrid analysis with Arnt fused to GAL4DBD was performed as described earlier. To find out which domains of Arnt mediate the interaction with Aint/Tacc3, several constructs of Arnt fused to GAL4DBD were assayed for interaction with full-length Aint in the two-hybrid system. The recombinant vectors containing PCR-amplified Arnt fragments are described in paper V. Two hybrid system and glutathione S-transferase (GST) pull-down experiments

were performed to study the interactions between $Aint_C$ (aa 307-631) and bHLH-PAS family members Arnt, Arnt2, AhR, Hif-1α and Sim2. The respective recombinant vectors were created as described in paper V. The inserts of these vectors were transcribed and translated in reticulocyte lysate (Promega, Madison, WI). Bacteria (BL21) containing pGEX- $Aint_C$ were grown and protein expression induced following the protocol described in paper V. Bacteria were lysed with PBS-containing solution (in detail in paper V) and centrifuged. The supernatant was loaded on glutathione-sepharose beads, mixed for 30 minutes at room temperature and then washed several times with PBS. GST- $AINT_C$-bound beads were incubated with *in vitro* expressed ^{35}S-labeled proteins as described in paper V. Beads were washed prior to analysis by sodium dodecyl sulfate (SDS)-polyacryl; amide gel electrophoresis. Input of 50 % of each radiolabeled protein was used for comparison.

4.3 Cell culture and transfection studies (Paper V)

To study the effects of Aint on HRE mediated transcription, endogenous Arnt and Hif-1α containing Hepa1c1c7 cells (cultured as described previously, Sadek and Allen-Hoffmann 1994) were transfected with a reporter plasmid containing three tandem repeats of the erythropoietin HRE upstream of the luciferase reporter gene (pHRE-luc was a gift from L. Poellinger) and with either empty vector pSG5 or pSG5Aint/Tacc3 (the vectors created as described in paper V). Cells were treated with iron chelatin hypoxia mimics, desferoxamine mesylate (DFX) or 2,2´-dipyridol (DP) as described in paper V.

Aint/Tacc3 fused to green fluorescent protein (GFP-Aint) was expressed in Hepa1c1c7 cells according to the protocol described in paper V in order to examine subcellular localization of Aint. The transfected cells were either left untreated as controls, or treated with DFX or TCDD. Cells were fixed with 3% paraformaldehyde in 5% sucrose/PBS and permeabilized with 0.1% Tween-20. A commercially available monoclonal antibody to Arnt (clone 2B10, IgG_1, ABR) was used as a primary antibody and a rhodamine-conjugated antibody as a secondary antibody (Jackson Labs, Bar Harbor, Maine).

5. GENE EXPRESSION EXPERIMENTS

5.1 RNA blot analysis

Northern blot analysis of whole embryo and postnatal mouse (E9 including placenta, E11, E13, E15, E17 and P1.5) mRNA was carried out as described in paper V using ^{32}P-labeled cDNA probes to full-length Aint/Tacc3 and GADPH (control) and Redi-Prime II kit (Amersham Pharmacia Biotech, Uppsala Sweden).

5.2 Radioactive *in situ* hybridization (I-VI)

To examine the localization of *Foxf1/Freac1, Foxf2/Freac2, Foxe3, Arnt/Arnt2* and *Aint/Tacc3* mRNAs the *in situ* hybridization was employed.

5.2.1 Probes

All the probes used in this study were 30-50-mer oligonucleotides (GC content 50-64%), which exhibited less than 70% homology to any other sequence when compared against the known sequences in GenBank database. At least two different probes were used to ascertain the specificity of hybridization. The probes were labeled at the 3´-end with α-^{33}P-dATP

(Du-Pont-NEN, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham Int., Buckinghamshire, UK). Probes with the same length, similar GC content and specific activity were used as controls.

TABLE 3. Sequences of the oligonucleotide probes used in this study

Gene	Sequences	Original reference
Foxf1/ FREAC1	5′gaggtggttgaagcccagcccgttcaccatgctgtacacaggcttgag 3′ 5′gggcaaagccatgccgtccacgttgccagccaaatggccattcatcat 3′	Hellqvist et al. 1996
Foxf2	5′ctcgggagacctggtaagggccactactggaccatcgacccggccagc3′ 5′gccacgcgcatccacagcacctccaccaccaccacgtcccccacatgt3′	Miura et al. 1998
Foxe3	5′ctcaggctgcaagcccaacaggctgtccaggcggaagag3′ 5′cagggaaggcttagccccaagcaaggctcggggacccagcgaattg3′	Blixt et al. 2000
Arnt	5′ctttcctaagagctcctgtggctggtagccaacagtagccacaca3′ 5′gcgtaagatggttagcttgtctggttttcgagccagggcactacagg3′	Carver et al. 1994
Arnt2	5′ggtgaggaggcagctgggctggagagaggactgtaggatgaagggt 3′ 5′cgctttcctcctcgggcaggcatggcccctgccattctcacctgtcc3′ 5′ccgctctgctgtccgtgatgctggctctgccactgggaccagacctc3′	Hirose et al. 1996
Aint/Tacc3	5′ggatcccgaggtggtgtctggaacgtcaca3′ 5′atggatcttcagcgcctggtacctctggcc3′	Sadek et al. 2000

5.2.2 Tissue preparation and hybridization

After decapitation of the pregnant female rats and mice, whole embryos were excised and frozen on a block of dry ice. After decapitation, P1.5 puppies were either frozen whole or dissected according to the tissues. After decapitation of older postnatal and adult animals, the tissues were excised and frozen. Serial 14 μm thick sections were cut with a Microm HM-500 cryostat and the sections were thawed on Probe On (Fischer Scientific, Pennsylvania, USA) or Polysine (Menzel-Gläser, Germany) glasses.

In situ hybridization was carried out as previously described in detail by Dagerlind and colleagues (1992), and Kononen and Pelto-Huikko (1997). The frozen sections were briefly air dried and hybridized at 42ºC for 18 hours with 5 ng/ml of the probe in a mixture containing 4 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 1 x Denhardt's solution (0.02% polyvinolpyrrolidone, 0.02% bovine serum albumine and 0.02% Ficoll), 1% sarkosyl, 0.02 M phosphate buffer (pH 7.0) and 10% dextran sulphate. After hybridization, the sections were rinsed 4 times at 55ºC in 1 x SSC for 15 min each and subsequently left to cool for 1 hour at room temperature. The sections were dipped in distilled water, dehydrated with 60 and 90% ethanol and air dried. Thereafter, the sections were covered with Kodak MR5 autoradiography film (Kodak, Rochester, NY) and exposed at -20ºC for 30 to 60 days. The autoradiography films were developed using LX24 developer and AL4 fixative (Kodak, Rochester, NY). Alternatively, the sections were dipped in NTB2 emulsion (Kodak) diluted 1:1 with distilled water and exposed at +4ºC. After 60days exposure the sections were developed with D19 developer (Kodak), fixed in G333 fixative (Agfa Gevaert, Leverkusen, FRG) and counterstained with hematoxylin-eosin or cresyl violet.

5.2.3 Image processing

The film autoradiograms were digitized with Nikon CoolScan II filmscanner. Emulsion dipped sections were analyzed using a Nikon FXA microscope equipped with an epipolarisation filter and SensiCam digital camera (PCO, Computer Optics GmbH, Kelheim, Germany) and IBM-PC. Images were processed using CorelDraw software (Corel Corporation Ltd., Ontario, Canada).

5.3 Non-radioactive *in situ* hybridization (Paper III)

Digoxigenin labeled antisense RNA probes were used for *in situ* hybridizations of cryosections as previously described (Henrique et al. 1995). Plasmids used to generate probes for *Pdgfra, Mki67* and *Crya1* were provided by Dr. C. Betsholtz, Dr. H. Igarashi and Dr. J. Piatigorsky, respectively. Probes for *Pcna* were generated from IMAGE cDNA clone No. 605791. Unique parts of *Cdkn1c* and *Prox1* were amplified by PCR from genomic mouse DNA and riboprobes were transcribed from promoters appended to the primers. The PCR products and cDNA clones were verified by DNA sequencing. The sense probes were used as controls.

5.4 Whole-mount *in situ* hybridization (III)

In situ hybridizations of whole mount mouse embryos were performed with digoxigenin-labeled antisense RNA probes also used for non-radioactive *in situ* hybridization according to the protocol previously described (Rosen and Beddington 1994).

6. IMMUNOCYTOCHEMISTRY (III,VI)

To reveal the immunoreactivity for Aint (Paper VI), light microscopic immunocytochemistry was performed. For that, whole embryos (E9, E13 and E16) and tissues of two days old (P2) mice were fixed by immersion in 4% paraformaldehyde in PBS for 3-6 hours. The tissue samples were cryoprotected with 15% sucrose in PBS, frozen with carbon dioxide, and 10 μm sections were cut. The embryos were embedded in paraffin, cut into 5 μm sections and deparaffinized. Subsequently paraffin sections were subjected to microwave antigen retrieval treatment as described earlier by Shi et al. (1991). Endogenous peroxidase activity was inhibited by immersing the sections in 0,5 % hydrogen peroxide in PBS for 20 min. The sections were first incubated with rabbit antibody raised against mouse Aint/Tacc3 protein at 4°C overnight in a buffer containing 1% BSA and 0,3% Triton X-100. After several washes the sections were incubated with biotinylated goat-anti-rabbit IgG and ABC complex (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) for 30 min, each. Nickel-intensified diaminobenzidine was used as a chromogen to visualize the sites expressing Aint/Tacc3-immunoreactivity. These sections were dehydrated and embedded in Entellan (Merck, Darmstadt, Germany). Controls included omission of the primary and secondary antibodies and presaturation of the primary antibodies with respective proteins. Only the reactions, which disappeared after presaturation of the antibody with the antigen were considered specific. In some organs, the antibody gave clear cytoplasmic staining which could not be prevented by presaturation and was thus regarded as nonspecific.

E-cadherin (Paper III) was detected with a rat monoclonal antibody (provided by Dr. H. Semb) and visualized with a FITC-conjugated secondary antibody (Dako, Copenhagen, Denmark).

7. DETECTION OF CELL PROLIFERATION AND APOPTOSIS (III)

In order to detect proliferative cells, BrdU incorporation was studied by using a kit from Roche Biochemicals (Indianapolis, IN) and fluorescence microscopy. 0.1 μmole of BrdU / g body weight was injected intraperitoneally to the pregnant females 4 hr before sacrificing to obtain *in vivo* labeling of embryos. To reveal the apoptic cells, TUNEL assay was performed with Roche Biochemicals (Indianapolis, IN) kit.

RESULTS

From this point onwards, the three forkhead genes *Foxf1 (FREAC1, HFH-8), Foxf2 (FREAC2, LUN)* and *Foxe3 (FREAC8)* are referred to only by their Fox names.

1. FOXF1 HAS A CELL-TYPE-SPECIFIC TRANSCRIPTIONAL ACTIVATION DOMAIN (I)

Earlier studies had obtained evidence for the existence of a cell-type-specific activation domain in FOXF1 (Hellqvist et al. 1996). Here, this phenomenon was studied more closely. First, transcriptional activation was shown to be dependent on the cellular context but to be promoter independent. The truncated FOXF1was a potent transcriptional activator in both tested lung-derived cell lines but it was inactive in two tested cell lines derived from other tissues (HeLa, human cervical carcinoma and COS-7, African green monkey kidney) whereas the full-length FOXF1 was a strong activator in all cell lines tested.

The C-terminal and N-terminal borders of the minimal region required for cell-type-specific activation domain were determined by transfection experiments. The region spans approximately 60 amino acids immediately downstream of the forkhead domain. The activation by this domain does not need the context of DNA-binding domain of FOXF1.

The removal of the most C-terminal 28 amino acids has been shown to switch the full-length FOXF1 from a strong transcriptional activator to an inactive protein (Hellqvist et al. 1996). The present deletion mapping tests confirmed that the function of this activation domain is independent of cellular context and it was called a general activation domain.

2. GENOMIC ORGANIZATION OF HUMAN *FOXF1* GENE (I)

FOXF1 has two exons separated by an 1.2 kb intron. The first exon encodes the DNA-binding domain and the cell-type-specific activation domain. The second exon encodes the general activation domain.

3. *FoxF1* AND *FoxF2* ARE EXPRESSED IN MESODERMAL TISSUES INVOLVED IN EPITHELIO-MESENCHYMAL INTERACTIONS (I, II)

FoxF1 and FoxF2 are closely related (Pierrou et al. 1994, Hellqvist et al. 1996, 1998). To acquire information concerning their distinct biological functions, the expression of *FoxF1* and *FoxF2* mRNAs were examined in mouse and rat embryos by *in situ* hybridization. *Foxf2* transcripts were examined also in postnatal and adult mice.

FoxF1 and *FoxF2* expression were detected at all times studied. No significant differences were observed between mouse and rat concerning *FoxF1* and *FoxF2* expression. The main sites of both *FoxF1* and *FoxF2* mRNA expressions were respiratory, alimentary and urinary tract where the signal was seen in the mesenchyme just beneath the endodermal epithelium. The epithelium itself did not express either *FoxF1* or *FoxF2*. In addition to common

expression sites, *FoxF2* was also expressed in the developing central nervous system, eye, ear and limbs.

The lung was the site of the strongest expression of *FoxF1* whereas the *FoxF2* expression was at the highest level in the intestines. In respiratory tract, both *FoxF1* and *FoxF2* mRNA expression were seen in the mesenchymal cells beneath the bronchial, tracheal, laryngeal and pharyngeal epithelium during embryonic development. In the lung, the expression of *Foxf2* remained high until P14 and a low signal was still observed in the adult.

High levels of *FoxF1* and *FoxF2* mRNA were seen throughout the alimentary canal at all embryonic times studied. In intestines, the expression of both *FoxF1* and *FoxF2* were observed in the mesenchyme of intestinal wall but their distribution was different. *FoxF2* was more evenly expressed whereas *FoxF1* was expressed more intensely immediately adjacent to the epithelium and mesothelium. No expression of *FoxF1* or *FoxF2* could be seen either in epithelium or mesothelium. Low expression of *FoxF1* was seen in the liver capsule whereas no *FoxF2* expression was seen in that area. In adult alimentary tract, a low signal for *Foxf2* was present only in the innermost part of circular muscle layer.

In developing teeth, the expression of both *FoxF1* and *FoxF2* was localized to the mesenchyme lining the tooth germ – not between inner and outer enamel epithelium as we erroneously reported in paper I. *FoxF1* expression was also observed in the dental pulp.

FoxF2 mRNA levels were higher in urinary tract than those of *FoxF1*. In the urinary bladder, there was a difference in the distribution of *FoxF1* and *FoxF2* similar to that seen in the intestine: *FoxF1* was present beneath both the epithelium and mesothelium, whereas *FoxF2* was expressed only adjacent to the epithelium.

A clear difference between the expression patterns of *FoxF1* and *FoxF2* was that only *FoxF2* mRNA was evident in the central nervous system. *FoxF2* mRNA was widely expressed in the neuroepithelium of the neural tube of E9 and E10 mouse embryo. During the later embryonic development, the expression was localized to many proliferating and differentiating brain areas. Postnatally, the expression was still evident in many parts of central nervous system but not any more in the adult.

Another striking difference between *FoxF1* and *FoxF2* expression was the presence of *FoxF2* transcripts in eye and ear. In the eye, the *FoxF2* expression was localized to the uvea and continued from E13 (mouse) to adulthood. In the ear, the signal was detected from E13 to P1.5 in the mouse. Finally, *FoxF2* was expressed in the developing limbs in the zone of polarizing activity and progress zone whereas *FoxF1* was not.

The summary of comparison between the expression of *Foxf1* and *Foxf2* mRNAs can be seen in table 4.

TABLE 4. Comparison of embryonic expression of *Foxf1* and *Foxf2*

Region	*Foxf1*	*Foxf2*
Central nervous system	-	+
Alimentary canal	+	+
Teeth	+	+
Respiratory tract	+	+
Urinary tract	+	+
Intervertebral discs	+	+
Limb bud	-	+
Eye	-	+
Ear	-	+

4. CLONING AND SEQUENCING OF *Foxe3* (III)

The mouse homolog of human *FOXE3* was cloned in order to enable the locating of spatial and temporal expression. Sequencing of 7 kb around the forkhead motif revealed a single open reading frame of 864 nucleotides corresponding to a 288-amino-acid protein.

5. *Foxe3* IS CRITICAL FOR LENS DEVELOPMENT (III)

5.1 Expression of *Foxe3* in mouse embryo

Foxe3 was expressed in the lens of the developing eye. Whole mount *in situ* hybridization showed the expression in lens placode and caudal forebrain at E9.5. The expression was localized to the lens vesicle when this structure detached from the surface ectoderm. The expression was first evenly distributed throughout the vesicle, but later the *in situ* hybridization of lens sections revealed that the expression ceased in the posterior cells of lens vesicle as these cells started to differentiate to lens fibers. In contrast, the expression remained high in the anterior part of the vesicle. The level of *Foxe3* transcripts in the anterior undifferentiated cells of lens remained high throughout fetal development into adulthood.

The expression in the limited area of the most caudal and dorsolateral parts of the diencephalon was no longer detectable by E11.5 by *in situ* hybridization. In addition to the lens and forebrain, no other sites for expression of *Foxe3* could be detected.

5.2 Colocalization of *Foxe3* with dysgenetic lens (III)

The very restricted expression pattern and the early onset gave us a clue to search for the function of Foxe3 by investigating its possible associations with developmental lens defects. The mouse mutation *dysgenetic lens (dyl)* offered a candidate because of its chromosomal localization (chromosome 4, Sanyal and Hawkins 1979, Sanyal et al. 1986), which is syntenic with the known human localization of *FOXE3* in 1p32 (Larsson et al. 1995). With fluorescent *in situ* hybridization *Foxe3* was mapped to 4C7, which was in perfect agreement with the genetic mapping of *dyl.*

Furthermore, the entire coding region of *Foxe3* was sequenced from a homozygous *dyl* mouse. Comparison with the wild-type *Foxe3* sequence revealed that *dyl* mice have two mutations in the region encoding DNA-binding domain of *Foxe3*.

5.3 Expression of *Foxe3* in *dyl* mice (III)

The homozygous *dyl* mice exhibit smaller eye, corneal opacity, adhesion of the iris, cataractous degeneration, and extrusion of the lens nucleus and persistent lens-epithelium attachment (Sanyal and Hawkins 1979). The earliest sign of this defect is the failure of the lens vesicle to separate from the ectoderm.

In situ hybridization showed similar patterns of *Foxe3* expression in wild-type and *dyl* lens during early development. At later stages, the morphological changes of *dyl* lens made the comparison difficult.

5.4 Studies on *dyl* lens (III)

In order to find evidence for the role of Foxe3 in cell proliferation and survival in the lens, we studied the defects of *dyl* lenses. The morphological examination of *dyl* lenses suggested that the primary defect leading to a dramatic reduction in the number of secondary lens fibers is the failure of the lens epithelium to proliferate. BrdU incorporation showed intense proliferation of epithelial cells in wild-type lens but scarceness of replicating cells in E14.5 and E15.5 *dyl* lenses. The growth arrest of *dyl* epithelial lens cells was also revealed by the down-regulated expression of genes encoding proliferation associated antigens *Mki67* and *Pcna* (proliferating cell nuclear antigen). In the *dyl* mutant, both *Cdkn1c* (encoding an inhibitor of Cdks, cyclin-dependent kinases) and *Prox1* (encoding a homeodomain protein related to *Drosophila prospero*) were misexpressed in the posterior part of the lens epithelium where the most rapid growth normally occurs. Cdkn1c blocks the cell cycle progression directly by inhibiting Cdks and Prox1 indirectly by activating *Cdkn1c*. The misexpression of these genes suggests that the cells in the posterior part of *dyl* lens epithelium cease to proliferate and instead appear to differentiate prematurely.

The expression of *Crya1* encoding α-crystallin (a marker for lens fiber cells) was low in the epithelium and highest in the equatorial zone of wild type lens whereas the expression was even throughout the *dyl* lens except in the most anterior cells. The elevated expression of *Crya1* and the lack of E-cadherin (a marker for epithelial cells) in the posterior lens epithelium of the *dyl* lens indicated premature differentiation. *Pdgfra* encoding platelet-derived growth factor (PDGF) A is normally expressed in the lens epithelium but in the *dyl* lens the expression was downregulated suggesting that the lack of proliferation in the *dyl* lens epithelium is due to the lack of growth factor signaling. The presence of excessive apoptosis in the anterior lens cells of *dyl* mutants was shown by TUNEL assay.

6. Arnt IS WIDELY BUT NOT UBIQUITOUSLY EXPRESSED DURING MOUSE DEVELOPMENT (IV)

The spatial and regional expression of *Arnt* mRNA was studied in E9-E17 mouse embryos and in the postnatal mouse at the age of 1.5 days by *in situ* hybridization. *Arnt* mRNA was

shown to be widely, but not ubiquitously, expressed both in the central nervous system and in the peripheral organs during mouse embryonic and postnatal development. The cell types expressing *Arnt* mRNA originated from endodermal, ectodermal and mesodermal origin.

The expression in the central nervous system was strong at E9 in the neuroepithelium of the neural tube. As development advanced, expression was seen in the proliferating periventricular primary neuroepithelium and secondary germinal matrices. *Arnt* mRNA was also detected in differentiating fields of the brain and spinal cord. However, the intensity of expression decreased as the neurons stopped proliferation and started migration and differentiation. A strong expression was detected in dorsal root ganglia.

Arnt mRNA was also widely distributed in the digestive system. *Arnt* mRNA was observed throughout the alimentary canal at all time points studied. *Arnt* was also expressed in the tongue, submandibular salivary gland, teeth and liver. Expression in hepatocytes was prominent from E11 to E15 but thereafter the signal clearly weakened. High levels of *Arnt* mRNA were indicated in lung at E13 both in epithelial and mesenchymal cells. Thereafter, the levels diminished and were undetectable by P1.5.

A high expression of *Arnt* mRNA was seen in the kidney at E13 and a moderate expression still at P1.5. *Arnt* was also expressed in the genital tubercle, urinary bladder and urethra. Low levels of *Arnt* mRNA were seen in the heart and big blood vessels from E11-13 to P1.5. In the thymus, both cortical and medullary thymocytes were positive from E13 to P1.5. In the spleen, *Arnt* mRNA was detected from E17 to P1.5. The epidermis, hair follicles and vibrissae were labeled from E13 to P1.5.

Expression in limbs started already at E11 in the limb bud and continued until E17. Positive mesenchymal cells were indicated in the mesenchyme around the developing bones from E13 to E17. Expression was seen in the muscles from E13 to E15.

From E13 to P1.5 expression was detected in the eye and olfactory epithelial cells. *Arnt* mRNA was located both to the inner and outer nuclear layers of retina. In the inner ear, a weak expression was observed from E13 to E17 in the cochlear epithelial cells. The adrenal cortex exhibited a prominent expression from E13 to P1.5. *Arnt* transcripts were seen from E13 to P1.5 in the pituitary gland and at E17 in the brown fat. Moderate expression was seen on the embryonic side of the placenta and weak expression in the yolk sac at E9 and E10.

7. *Arnt2* HAS A DYNAMIC EXPRESSION PATTERN IN DEVELOPING CENTRAL NERVOUS SYSTEM AND ALSO IN PERIPHERAL TISSUES (IV)

The main site of *Arnt2* expression was the central nervous system, although the expression was widely seen also in the peripheral organs. The levels of expression were strongest in central nervous system at all time points studied. Outside the nervous system, the expression of *Arnt2* mRNA was more limited than that of *Arnt.*

Expression was very prominent already at E9 in the neuroepithelium of the neural tube. As the development advanced, *Arnt2* transcripts were seen in all neuronal areas including the periventricular primary neuroepithelium, secondary germinal matrices and also differentiating fields of the brain and spinal cord. The intensity of expression varied in different parts of the developing brain. Strong labeling was seen in the dorsal root ganglia and in the trigeminal ganglion.

Outside the central nervous system, a very strong signal was detected only in the retina, where expression was seen from E13 onwards and still postnatally. Both the inner and outer nuclear layer of retina expressed *Arnt2* mRNA. In digestive system, tongue expressed *Arnt2* mRNA during embryonic period but not any more postnatally. In the intestines, stomach and teeth, low to moderate expression was evident both during the embryonic period and postnatally. In the intestinal canal, the expression was located in all layers of the wall. In the salivary gland, expression was seen at E17 but the signal was undetectable postnatally. No signal for *Arnt2* mRNA was seen in the liver.

Both epithelial and mesenchymal cells of the lung expressed *Arnt2* during embryonic development but not postnatally. Strong expression was seen in the genital tubercule at E11 and E13. In the urethra, *Arnt2* mRNA was indicated in all layers at E15 and E17. In the urinary bladder the signal was located in the muscular layer and the transitional epithelial cells. A moderate level of *Arnt2* transcripts was seen in the kidney, in the epithelial cells of glomeruli from E13 to P1.5.

No *Arnt2* mRNA was detected in the heart. In the walls of large vessels a weak labeling was seen at E15 and E17. In spleen, *Arnt2* mRNA was observed at P1.5 and in thymus at E17 and P1.5. *Arnt2* transcripts were present in the epithelial cells of the most superficial layer of the epidermis perinatally. The follicles of vibrissae were positive from E13 to E17.

Arnt2 mRNA was observed in the developing musculoskeletal system during embryonic development but not postnatally. The expression was seen in the mesenchymal cells surrounding the developing bones including backbones, and in the progress zone in the distal tip of the limb. In muscle cells, the expression was seen at E13 and E15.

In the olfactory and cochlear epithelial cells a strong expression was indicated at E17 whereas the intensity of expression around that stage was lower. In the adrenal medulla, a strong signal was observed at E13 and at P1.5. The thyroid gland expressed *Arnt2* mRNA at E17 and the pituitary gland from E15 to P1.5. No signal was seen in adipose tissue. The expression of *Arnt2* in placenta differed clearly from that of *Arnt*, as *Arnt2* transcripts were seen (at E9 and E10) only on the maternal side of the placenta.

8. CLONING AND SEQUENCING OF *Aint/Tacc3* (V)

A novel 2.1 kb clone (accession no. AF156934) was isolated from mouse as a result of search for Arnt interacting proteins by yeast two-hybrid system from 17-day mouse embryo library. It was termed Aint (***A***rnt ***int***eracting protein). The screening of a 16-day mouse embryo cDNA library using the original *Aint* clone as a probe led to the isolation of 2.1 kb

insert. The predicted amino acid sequence of Aint contained an open reading frame of 631 amino acids including a series of seven repeats of 24 amino acids. The analysis of C-terminus revealed two coiled-coil regions which had homology to human transforming acidic coiled coil proteins TACC1 (Still et al. 1999a), TACC2/AZU1/ECTACC (Still et al. 1999a, Chen et al. 2000, Pu et al. 2001) and TACC3/ERIC1 (Still et al. 1999b, McKeveney et al. 2001), mouse Tacc3/Eric1 (AF203445/AF203446) (Still et al. 1999b, McKeveney et al. 2001), *Xenopus* maskin (Stebbins-Boaz et al., 1999) and *Drosophila* TACC (Gergely et al. 2000a). The coiled-coil region is composed of a myosin tail, which includes myosin molecules. These are multi-subunit complexes, contractile proteins, found in all eukaryote cell types and can assemble into the macromolecular thick filament (Strehler et al. 1986).

The homology with *Tacc3/Eric1* suggests that *Aint* and *Tacc3* are from the same gene but incomplete as they are shorter than that of Aint. For the simplicity of the nomenclature, the decision was made that Aint is called Tacc3 and this term is used from this forward.

9. THE BIOLOGICAL FUNCTIONS OF Tacc3 (V)

Yeast two hybrid interaction tests revealed that the region of Tacc3 responsible for interaction with Arnt is the C-terminus of the protein, which is the most conserved domain in TACC proteins. In Arnt, the interaction with Tacc3 was mediated via PAS domain. The C-terminal part of Tacc3 interacted also with Arnt2 but not with either AhR, regardless of the addition of a ligand, or Hif-1α. Interaction with mSim2 was minimal.

In GFP-Tacc3 transfected Hepa1c1c7 cells that contain endogenous Arnt, Tacc3 was localized mainly to cytosolic compartment. Tacc3 overexpression led to a non-nuclear localization of ARNT. After treating the cells with an iron chelating inducer of the Hif-1 pathway (deferoxamine mesylate) or an activator of AhR/ARNT pathway (TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin), nuclear localization of Arnt was restored to some extent.

Transfection experiments using Hepa1c1c7 cells containing endogenous Arnt and Hif-1α were performed to examine if Tacc3 is involved in Arnt/Hif-1α pathway. The cells transfected with Tacc3 had a reduced background activity on the HRE reporter. Treatment with chemical inducers of hypoxia response pathway led to an efficient induction of transcriptional activity on the HRE. The cells transfected with Tacc3 had a 1.5 – 2.5-fold increase in responsiveness compared to the cells transfected with plasmid alone.

10. Tacc3 IS EXPRESSED IN PROLIFERATING MOUSE TISSUES (V, VI)

10.1 Distribution of *Tacc3* transcripts in developing and adult mouse

Northern analysis of embryonic and postnatal whole mouse mRNA revealed the presence of *Tacc3* transcripts at all time points studied (E9, E11, E13, E15, E17 and P1.5). The highest levels were detected at E11, E13 and E15.

In agreement with the results of Northern blotting, *in situ* hybridization analysis showed the most widely spread and highest expression of *Tacc3* mRNA at E11, E13 and E15. In some

organs the expression continued postnatally. In adult, only testis and ovary expressed *Tacc3* at high level and thymus and spleen at low level.

At the earliest time points studied, at E9 and E10, *Tacc3* expression was strong in the neuroepithelium of neural tube, which is the rapidly growing primary proliferative matrix giving rise to all neural elements of the brain. From E11 to E17 the expression was seen both in the neuroepithelium and in the proliferative secondary germinal matrices surrounding the ventricular lumen and from E13 onwards in the external germinal layer of cerebellum. The differentiating neurons of brain parenchyma did not express *Tacc3* mRNA. At the perinatal period, expression started to weaken and cease in primary germinal matrices whereas in some subventricular zones a weak signal was seen a little longer after birth. In later developing cerebellum, the expression continued until two weeks after birth.

In the alimentary tract, *Tacc3* mRNA expression was evident at E11 in intestines and liver. In liver, most of the hepatocytes expressed *Tacc3* mRNA until E15. Thereafter, no signal could be detected. The intestinal expression was seen in all layers of the intestinal wall in every part of the canal until P1.5. In the tongue, both epithelial and muscle cells expressed *Tacc3* at E13 and E15 while the expression was confined to the epithelium at E17 and P1.5. In the tooth and salivary gland, the signal was seen until P1.5. Later than P1.5 no expression could be detected in alimentary tract by this method.

In respiratory tract, the expression was seen from E11 (in nasal process and lung) to E17 (in lung and trachea). The strongest expression was observed at E13 in lung mesenchymal and bronchial epithelial cells.

In the genito-urinary system, the expression was high at E13 in the genital tubercle and in the primitive cortical region of the kidney. A weak expression was seen in the medullary region. The mRNA levels in kidney decreased to undetectable by two weeks after birth. In the urinary bladder, a weak to moderate expression was detected from E13 to P1.5. The testis was studied at E17 and postnatally, and increasing expression levels reaching high level in adult were seen. Most of the seminiferous tubules exhibited strong signal in the adult. Expression was strong in primary and secondary spermatocytes and lower in early round spermatides. Ovary was studies at the age of 1 and 2 weeks and in adult. At all time points, oocytes of primordial, primary and secondary follicle stages exhibited strong expression whereas expression was weak in granulosa cells. Moderate expression was detected in the interstitial cell and the germinal epithelium at the age of 1 and 2 weeks.

Heart muscle and walls of the big vessels expressed *Tacc3* from E11-13 to P1.5. In lymphatic organs spleen and thymus, expression was seen both in the embryonic period and postnatally still in the adult. During embryonic development, both medullary and cortical thymocytes expressed *Tacc3* very strongly, whereas postnatally the transcripts were located abundantly in the outer cortex and to a lesser extent in the medulla. In the spleen, expression was moderate both in the white and red pulp from E17 to P3. In the adult, only the red pulp exhibited a low expression.

In skin, *Tacc3* mRNA expression was seen from E13 to P1.5. The signal was located in the epidermis and follicles of hair and vibrissae in the dermis. A moderate expression was observed in somites and in limb buds at E10 and in the branchial arches at E11. During embryonic development, ligaments, muscles, mesenchyme adjacent to the primordial bones and future intervertebral discs expressed *Tacc3.*

In the eye, in the inner nuclear layer of the retina, the period of *Tacc3* expression extended from E13 to the age of 2 weeks. The expression could be detected at E13 and E15 in mesenchymal cells of inner ear and from E13 to P1.5 in olfactory epithelial cells. In thyroid gland, *Tacc3* transcripts were seen at E17, in the adrenal gland at E15 and P1.5 and in the pituitary gland from E13 to P1.5. A low expression was observed in the brown fat at E17.

The placenta was studied at E9 and E10, and *Tacc3* mRNA expression was seen in all layers. In the yolk sac, moderate expression was observed.

10.2 Tacc3 immunoreactivity in embryonic and postnatal mouse (VI)

Tacc3 immunoreactivity (IR) was studied at E9, E13, E16 and P2. Tacc3-IR was nuclear and often localized to distinct dots in the nucleus. No specific cytoplasmic staining was seen. At E9, Tacc3-IR was detected widely in the mesenchymal cells. At this stage most of the neuroblasts of the neuroepithelium of neural tube exhibited Tacc3-IR. The epithelium of the developing intestinal canal and the surrounding mesenchymal cells expressed Tacc3.

In the central nervous system, from E13 to E16 the strongest Tacc3-IR was seen in the proliferating neocortical neuroepithelium and in subventricular zone surrounding the lateral ventricles. A weaker reaction was seen in the nuclei of the neuroblasts of the differentiating field. At E16 Tacc3-IR was detected in cerebellar neuroepithelium and the external germinal layer. Also ependyma of choroid plexus was positive. At P2 a strong Tacc3-IR was seen in the thalamic nuclei, hippocampus, meninges, ependyma and in external germinal layer of cerebellum. Separate stained cells were also observed in differentiating field. In the peripheral nerves, the nuclei of Schwann cells were positive.

In the intestinal canal, Tacc3-IR was detected at all times studied. At E13, the intestinal epithelia were negative while a weak staining was seen in muscle cells of the intestinal wall. However, at E16 and P2 also epithelial cells in addition to cells of the muscle layer and lamina propria showed Tacc3-IR. At E13 and E16, the majority of the hepatocytes exhibited Tacc3-IR. At P2, Tacc3-IR in the liver had diminished and only a minority of the hepatocytes were positive. Moderately stained granulocytes and lymphocytes were also detected in the liver. Tacc3-IR was evident in the bile duct and the gall bladder where the staining was located to columnar epithelial cells. In the pancreas, at E13 mesenchymal cell exhibited Tacc3-IR whereas the acinar cells were negative. By E16 the expression had changed and Tacc3-IR was seen in the nuclei of the pancreatic acinar cells. Still at P2, the acinar cells were positive. In the salivary gland, Tacc3-IR was observed at E16 both in epithelial and mesenchymal cells. In lung, Tacc3-IR was seen in mesenchymal cells at E13 and E16 and in bronchial epithelial cells at E16 but not at E13.

Epithelial cells covering the genital tubercle were strongly stained at E13. In the kidney, Tacc3-IR was evident at P2 in the epithelium of the collecting tubules and the loops of Henle, in the glomerular podocytes and in the transitional epithelial cells of the renal pelvis. At P2, both the ovary and testes showed Tacc3-IR. In the ovary, strong labeling was observed in oocytes, granulosa cells of primordial follicles, interstitial cells and cells of germinal epithelium. In the testes, positive cells were Sertoli cells, primitive germ cells, interstitial cells, peritubular cells and fibroblasts of tunica albuginea.

Tacc3-IR was seen in the muscle cells of heart and in the endothelial cells of the vessels at E13 and E16. A large number of thymocytes was stained at E13 and E16. At P2 Tacc3-IR had strengthened in the cortical thymocytes of the outermost layers of the cortex. Also the epithelio-reticular cells and thymocytes of the medulla were weakly positive. In the spleen, some lymphocytes were moderately stained at P2.

In future intervertebral discs, Tacc3-IR was observed at E13. At E16 a strong Tacc3-IR was observed in muscle cells. In the organs of special sense, Tacc3-IR was detected in the olfactory epithelium at E13, in the cochlear epithelium at E16 and in the retina at E16 and P2. In the eye Tacc3-IR was first seen in the inner layer and later both in the inner and outer layer. Also corpus ciliare and epithelial cells of Hardenian glands exhibited Tacc3-IR. In the skin, expression was seen in the basal layer of the epidermis, hair follicles and the striated muscle cells. Tacc3-IR was observed in the brown fat at E16 and in adipocytes at P2. In the thyroid gland, Tacc3-IR was detected at E16. All layers of the placenta (at E9) showed Tacc3-IR. A moderate staining was seen in the yolk sac.

DISCUSSION

1. REGULATION OF *Foxf1, Foxf2* AND *Foxe3* IN THE DEVELOPING RODENT

The forkhead proteins FOXF1 and FOXF2 are closely related. In addition to the almost identical primary structures of their DNA-binding motifs they are also similar in the C-terminus (Hellqvist et al. 1996). Both proteins are transcriptional activators (Hellqvist et al. 1996).

FOXF1 was expected to have a cell-type-specific activation domain (Hellqvist et al. 1996). In paper I, the transcriptional activation domains of FOXF1 were studied more closely. A cell-type-specific activation domain acting in the lung-derived cell lines was localized to the central part of the protein and a general activation domain was confirmed to reside in the C-terminal end. Activation by the cell-type-specific activation domain was promoter independent and did not require the context of the DNA-binding domain.

As the function of the activation domain in the central part of FOXF1 depends on its cellular context, its mode of function differs from that of most other activation domains. FOXF1 binds DNA as monomer and this excludes dimerization with alternative partners and cannot explain the differences in activation properties between cell lines. Neither does competition with endogenous FOXF1 offer an explanation, since none of the cell lines used express endogenous FOXF1. The cell-type-specific activation domain is likely to involve a specific adapter or coactivator proteins present in certain cells. To discover these possible interaction proteins will be an interesting topic for further studies.

Like FOXF1, FOXF2 has two activation domains (Hellqvist et al. 1998). Activation domain 1 in the C-terminal end of FOXF2 shares the sequence similarity with the general activation domain of FOXF1 whereas the activation domain 2 is formed by three synergistic subdomains in the central part of the protein and shows no obvious homology to other known proteins (Hellqvist et al. 1998).

The genomic structure of *FOXF1* appeared to be very similar to that of previously defined *FOXF2* (Blixt et al. 1998). Both genes consist of two exons separated by an intron. However, these two genes are located on the different chromosomes.

Foxf1 and *Foxf2* mRNA expression was studied in mouse and rat embryos and *Foxf2* also in postnatal and adult mice. Consistent with their structural and functional similarities, the expression patterns exhibited several congruencies: the main sites of expression during embryonic development for both genes were respiratory, alimentary and urinary tracts. Both transcripts were found in mesenchyme adjacent to the epithelia, whereas the epithelial cells did not express the mRNAs. No differences between separate species were found.

In common expression sites, these transcriptional activators may have overlapping functions in epitheliomesenchymal cross-talk. However, several qualitative and quantitative differences were found, also suggesting separate roles as developmental regulators. *Foxf2* appeared to be more widely expressed than *Foxf1* (Table 4). Its distinct expression in the

developing central nervous system, eye, ear and limb buds suggests a developmental role independent of *Foxf1* in these organs.

The recent study which presented the targeted mutation of *Foxf1* confirms the importance of Foxf1 for murine development (Mahlapuu et al. 2001a). Mutant embryos die at midgestation due to defects in mesodermal differentiation and cell adhesion. The embryos fail to turn and become deformed because of the small and inflexible amnion. In the yolk sac and allantois, no vasculogenesis occurs, chorioallantoic fusion fails and the amnion misexpresses vascular and hematopoietic markers. Mahlapuu and the co-workers (2001a) also reported the expression of *Foxf1* during earlier murine embryogenesis than what was studied in paper I. At E8.5, *Foxf1* transcripts were localized in the mesoderm of the posterior primitive streak, lateral plate and the extra-embryonic mesoderm of allantois and yolk sac consistent with the consequences of the gene disruption. Earlier, Peterson et al. (1997) had detected *Foxf1 (HFH-8)* in the amnion in agreement with the failure of this structure to develop normally in knockout animals.

By analysis of *Foxf1* heterozygotes, Mahlapuu et al. (2001b) demonstrated the importance of *Foxf1* for normal foregut and lung development as could be expected on the basis of the strong expression in these sites described in paper I. The *Foxf1* +/- mutants exhibited various malformations as lung immaturity and hypoplasia, fusion of the right lung lobes, narrowing of the esophagus and trachea, esophageal atresia and tracheo-esophageal fistula. (Mahlapuu et al. 2001b). Although Foxf1 and Foxf2 share many similarities (structure, transcriptional activation, target specificity), the phenotypes of both *Foxf1* homozygotes and heterozygotes propose that in affected organs and structures, the compensation by *Foxf2* is lacking or insufficient. Foxf2 -/- mutants die perinatally (Miura et al., personal communication) but their exact phenotype has not yet been published. In the future, the targeted and inducible mutations of *Foxf1* and *Foxf2* will shed light on the diverse and shared developmental functions of Foxf1 and Foxf2. It will be interesting to see whether the failures caused by lack of Foxf2 are at the same or different sites where Foxf1 is most important.

Foxf2 expression is mainly restricted to fetal development. Within two postnatal weeks, detectable expression ceased in all other organs studied except in the eye, stomach, intestines and lung, where expression was seen also in the adult. For example, in the lung both *Foxf1* and *Foxf2* transcripts were seen during the entire fetal development. High levels of *Foxf2* were seen two weeks postnatally and the expression had decreased to low level in the adult. Our observation of *Foxf2* expression in adult lung differs from that reported by Miura et al. (1998). Miura et al. detected *Foxf2* mRNA in the adult mouse lung in agreement with our results but they identified the cell types responsible for the expression as bronchiolar epithelial cells and type II pneumocytes. Both of these epithelial cell types are of endodermal origin. We observed the expression in the lung only in mesodermally derived cells. Our results of *Foxf2* expression resemble that of *Foxf1* in the adult lung reported by Peterson et al. (1997) and Kalinichenko et al. (2001).

Based on the expression pattern where the transcripts are found in mesenchyme adjacent to epithelium we proposed that *Foxf1* and *Foxf2* are activated by paracrine signals from

endodermal epithelia. This was later confirmed concerning *Foxf1* by the study of Mahlapuu et al. (2001b). *Foxf1* transcription was shown to be activated by Sonic hedgehog (Shh) signaling. No *Foxf1* expression could be seen in *Shh* -/- embryos and, on the other hand, the ectopic secretion of Shh in *in vitro* wild type lung explants activated *Foxf1* expression in the lung mesenchyme. Growth factors FGF7 and FGF10 downregulated *Foxf1* and this was speculated to be mediated by *Bmp4*, that also decreased *Foxf1* expression when applied locally (Mahlapuu et al. 2001b). The open question is still, which are the downstream genes regulated by *Foxf1*. Another subject for further studies is the search for other possible epithelial inducers for *Foxf1* in addition to Shh. Further, it will be interesting to find out the inducers for *Foxf2*.

The expression of *FoxF1* and *FoxF2* mRNA was studied in both the developing mouse and rat to find out possible differences between species. No significant divergences were found suggesting equivalent biological tasks for Foxf1 and Foxf2 in these rodents and possibly more widely in mammals.

In paper III, evidence was provided that a novel forkhead gene, *Foxe3*, plays an important role in lens formation. Lens formation is a classical model of induction (Speman 1901), which nowadays is known to be a multi-step process (Grainger 1992). Several transcription factors, e.g. Lhx2, Rx, Six3, Prox1 and Pax6, have been shown to be essential for lens development (reviewed by Chow and Lang 2001). We showed that *Foxe3* was expressed in the early stages of lens induction and the expression became confined to the proliferating cells of the anterior lens epithelium and was turned off in differentiating fiber cells. In the undifferentiated cells of the anterior surface of the lens, the expression was maintained still in the adult. In addition to the lens, the only expression site observed was in a limited area of the brain during a short period of embryonic development (E9.5 – E11). Also these cells represent relatively undifferentiated type.

We provided strong evidence that the mutations in *Foxe3* cause the phenotype of the classic mutant *dysgenetic lens*. First, the defects in *dyl* mice appear in sites where *Foxe3* is expressed. Second, the chromosomal location of mouse *Foxe3* is in agreement with the region where *dyl* had been mapped previously in the vicinity of *dysgenetic lens* locus (Sanya et al. 1986). Third, *dyl* mice have two mutations in the part of *Foxe3* encoding the DNA binding domain and these mutations are likely to disrupt *Foxe3* function by disturbing the DNA-binding ability of the protein. Last, the identified mutations in *Foxe3* cosegregate with the *dyl* phenotype and are therefore likely to cause the mutant phenotype. The results concerning the connection between *Foxe3* and *dyl* phenotype were confirmed by Brownell et al. (2000) in a work that was published soon after our report.

In *dyl* mice, the most impressive feature is the tenacious connection between the lens and corneal epithelium which is due to the failure of the lens vesicle to close and detach from the ectoderm. The primary cellular defect is the failure of the lens epithelium to proliferate and presumably mutation in *Foxe3* gene is responsible for that. The expression pattern restricted to the undifferentiated cells suggests a role for *Foxe3* in anti-differentiation mechanisms. It may promote survival, and proliferation of lens epithelial cells while preventing differentiation. Foxe3 possibly prevents the expression of genes involved in

inhibition of growth and in differentiation. *Cdkn1c* and *Prox1* are among these inhibitory genes and they indeed exhibited an altered expression in *dyl* lens cells (Zhang et al. 1998, Wigle et al. 1999). As a consequence, the normal rapid growth of anterior epithelial cells was changed to inhibition of growth and finally to programmed cell death as demonstrated in our work. In posterior part of *dyl* lens, the cells ended up to differentiate prematurely. Because the inhibitory mechanism of Prox1 is mediated by activation of *Cdkn1c*, *Foxe3* may function as a repressor of *Prox1* expression (Wigle et al. 1999).

Brownell et al. (2000) studied *Foxe3* expression in *Pax6 -/- (Small eye)* and *Rx -/-* embryos which lack lens formation. In *Rx -/-* embryos, *Foxe3* was not expressed in the surface ectoderm although the expression in the brain was not affected. In *Small eye* mutant embryos the lack of *Foxe3* was observed in the superficial head ectoderm and greatly reduced expression in the brain. These results place *Foxe3* activity downstream from *Pax6* and *Rx*.

The expression of a growth factor receptor gene (*Pdgfra)* was diminished in *dyl* mutants while highly expressed in wild-type lens epithelium (Orr-Urtreger and Lonai 1992). The ligand, PDGF A, has previously been shown to stimulate growth of lens epithelium and the *Patch/Patch* mouse embryos homozygous for a null mutation in *Pdgfra* gene exhibit reduced number of secondary fibers in the lens (Morrison-Graham et al. 1992). Therefore, it is probable that this growth factor signaling depends on *Foxe3* activation, although, other growth factors are presumably also involved. In future, the role of *Foxe3* in the pathways responsible for controlling lens development could be studied by transgenic mice overexpressing *Foxe3*. The inducible knock-out might reveal the function of *Foxe3* in the adult.

After we had shown the crucial role of *Foxe3* for lens development in the mouse, the human homolog *FOXE3* was found (Semina et al. 2001). *FOXE3* expression was detected in the anterior epithelium of the adult human lens and it was shown to be mutated in patients with anterior segment dysgenesis and cataracts (Semina et al. 2001).

Several oncogenic Fox proteins have been found (reviewed by Vogt et al. 1997). So far, no associations with tumorigenesis have been revealed concerning FOXF1, FOXF2 or FOXE3, although there are two putative AML1 (acute myeloid leukemia) binding motifs in *Foxf1a* (Chang and Ho 2001).

2. Arnt AND Arnt2 MAY HAVE PARTLY OVERLAPPING AND PARTLY INDEPENDENT ROLES IN MURINE DEVELOPMENT

Arnt1 and Arnt2 have a special role among the members of bHLH-PAS protein family as they interact with other family members and help in transducing signals between environment and a transcriptional machinery of the cell (Rowlands and Gustafsson 1997, Gu et al 2000). They have also been shown to interact with non-related partners (Carver and Bradfield 1997, paper V). The experiments performed with knockout models have proved their importance for embryonic development and it can be suggested that the mechanisms of environmental adaptation are also used in developmental processes (Gu et al. 2000). The

studies showing the gene expression of *Arnt* and *Arnt2* have been superficial and the aim of our work was to obtain detailed information concerning the distribution of *Arnt* and *Arnt2* mRNA during embryonic and early postnatal development.

We were able to reveal several new sites of expression for *Arnt* and especially for *Arnt2*, which in previous studies had been shown to have a rather restricted expression pattern (Hirose et al. 1996, Jain et al. 1998). However, our study mostly confirmed the results of earlier experiments, for the previously reported sites exhibited the most intense expression in our research also. Both genes were expressed at all the times studied and showed both temporal and spatial specificity.

The developmental signals may be functionally similar to environmental stresses and we can consider the developing embryo as an organism adapting to the environmental challenges appointed by multicellularity (Gu et al. 2000). Next, two models of bHLH-PAS dependent response pathways (the aryl hydrocarbon receptor pathway and hypoxia signaling pathway) will be discussed regarding the results of our study and the possible roles of Arnt and Arnt2 in developing mouse embryo.

The aryl hydrocarbon receptor pathway allows animals to adapt to adverse chemicals by regulating xenobiotic metabolizing enzymes in order to decrease the biological half-life of detrimental chemical (Rowlands and Gustafsson 1997, Crews 1998, Gonzalez and Fernandez-Salguero 1998, Gu et al. 2000). AhR and Arnt are known to be crucial proteins for this adaptive mechanism and Arnt2 may have a role as an alternate partner for the AhR (Hirose et al. 1996). Also it has been suggested that the developing organism has a developmental requirement for this pathway, possibly due to some unavoidable or endogenous toxicant or because the receptor system plays other roles in addition to its known adaptive functions (Gu et al. 2000, Poellinger 2000). This theory is supported by the gene targeting experiments in mice, which show the importance of *AhR*, *Arnt* and *Arnt2* for mouse development (Fernandez-Salguero et al. 1995, Schmidt et al. 1996, Kozak et al. 1997, Maltepe et al. 1997, Mimura et al. 1997, Hosoya et al. 2001, Keith et al. 2001). In addition, a critical endogenous role for AhR is also proposed on the basis of results indicating the existence of AhR in the nucleus of HeLa cells in the absence of exogenous ligand (Singh et al. 1996). The AhR-null mice have been shown to be resistant to the acute toxicity of TCDD and to have an altered teratogenic response to that compound (Gonzalez and Fernandez-Salguero 1998).

The results of our work and previous studies mapping the developmental expression of *AhR*, *Arnt* and *Arnt2* reveal the sites where AhR-Arnt and AhR-Arnt2 dimers could be formed (Abbott and Probst 1995, Abbott et al. 1995, Hirose et al. 1996, Jain et al. 1998). *AhR* and *Arnt* are parallelly expressed in several developing tissues including the branchial arches, oral cavity, tongue, intestines, liver, lung, genital tubercle, kidney, urinary bladder, heart, thymus, muscles, retina, adrenal gland, pituitary gland and neuroepithelium. Most of those sites were also shared by *Arnt2* transcripts, except the liver and heart. Interestingly, among the phenotypes reported for $AhR^{-/-}$ mice are a small liver size and portal fibrosis (Fernandez-Salguero et al. 1995, Schmidt et al. 1996) indicating an important physiological role for AhR in normal liver development. If the whole AhR pathway is needed for liver

development, Arnt may well be the interaction partner for AhR in liver organogenesis in the mouse according to the results of our and previous reports (Abbott et al. 1995, Jain et al. 1998). In most of the peripheral organs where all three genes are expressed, there are no clear differences between the expression of *Arnt* and *Arnt2* and they might have compensatory roles as partners in the aryl hydrocarbon receptor pathway. In the central nervous system *AhR*$^{-/-}$ mice exhibited no detectable malformations and the role of AhR pathway seems not be crucial for brain development (Fernandez-Salguero et al. 1995, Schmidt et al. 1996, Mimura et al. 1997). Instead, the prominent defects in the central nervous system of *Arnt*$^{-/-}$ and *Arnt2*$^{-/-}$ embryos are mediated by other mechanisms (Kozak et al. 1997, Maltepe et al. 1997, Hosoya et al. 2001, Keith et al. 2001).

The AhR pathways have been suggested to participate with growth factors and hormone signaling mechanisms in the cell cycle control (Zaher et al. 1998, Gonzalez and Fernandez-Salguero 1998). The interaction with retinoic acid/TGFβ may be involved with the possible endogenous role of AhR (Zaher et al. 1998). In conclusion, the precise role of the AhR pathway in embryonic development in mammals is unclear and it would be important to find an endogenous ligand or possible alternative mechanisms which activate AhR.

The hypoxia response pathway involves several bHLH-PAS proteins that function as sensors (e.g. Hif-1α, Hif-2α/EPAS1/MOP2 and Hif-3α or as partners (Arnt, Arnt2 and Arnt3) (Bunn and Poyton 1996, Gu et al. 2000). This pathway helps an organism to adapt to hypoxia, which is also an important signal for normal development (Gu et al. 2000). The parallel expression of Arnt, Arnt2 and Hif-1α, which permits the interaction, is observed in the neuroepithelium, branchial arch, intestines, kidney, thymus and olfactory epithelium (Jain et al. 1998). In addition, Arnt and Hif-1α are seen in the liver and heart of mice embryos. However, we have to keep in mind that preconditions for the dimer formation between Arnt and Hif-1α and Arnt2 and Hif-1α are that mRNAs are translated and that the proteins really exist in the same cells, which have not been proved so far.

Gene inactivation models have shed light on the implication of the hypoxia signaling pathway for murine development. First, *Arnt*$^{-/-}$ embryos which displayed lethality between E9.5 and E10.5 had major defects in angiogenesis and also in central nervous system development (Kozak et al. 1997, Maltepe et al. 1997). Second, *Hif-1α*$^{-/-}$ embryos died at E11 and exhibited a phenotype rather similar to *Arnt*$^{-/-}$ embryos with neural tube defects, cardiovascular malformations, and a lack of cephalic vascularization (Iyer et al. 1998, Ryan et al. 1998, Kotch et al. 1999, Semenza et al. 1999). *Hif-1α*$^{-/-}$ mutants were also lacking the enzymatic responses for hypoxia (Iyer et al. 1998, Ryan et al. 1998). The phenotypes of *Arnt*$^{-/-}$ and *Hif-1α*$^{-/-}$ mouse embryos support the importance of a hypoxic signal for normal development. The Hif-1α/Arnt-dimer formation is presumably essential for transcriptional activation of genes involved in developmental angiogenesis. However, the vascular malformations of *Hif-1α*$^{-/-}$ mutant embryos were more severe than those of *Arnt*$^{-/-}$ mutants at E9.5 suggesting that the loss of Arnt can be partly compensated by other proteins.

Also Arnt2 has been shown to form complexes with Hif-1α (Drutel et al. 1996, Hirose et al. 1996). *Arnt2*$^{-/-}$ mice die perinatally and exhibit impaired hypothalamic development (Hosoya et al. 2001 and Keith et al. 2001). A very similar phenotype was earlier found in

Sim1$^{-/-}$ embryos supporting the assumption that these two proteins form a functional dimer *in vivo* (Michaud et al. 2000). Interestingly, we found a very strong expression of Arnt2 both in the hypothalamic neuroepithelium and in the hypothalamic differentiating field whereas the expression of Arnt was also strong in the neuroepithelium but only weak in differentiating cells. It is possible that the detected amount of *Arnt* mRNA is either insufficient to compensate for the lack of *Arnt2* in mutant embryos or the translation does not occur. The cultured *Arnt2*$^{-/-}$ neurons were shown to have a decreased hypoxic induction suggesting that also Hif-1α/Arnt2-dimers are needed for regulation of oxygen-responsive genes during development (Keith et al. 2001).

Our demonstration of partly overlapping expression patterns of *Arnt* and *Arnt2* mRNA supports the study of Keith et al. (2001), which suggests that Arnt and Arnt2 have a strong genetic interaction shown by crossed compound heterozygous mutant *Arnt*$^{+/-}$, *Arnt2*$^{+/-}$ mice embryos. In our study, we presented a much wider expression pattern for Arnt2 than the previous reports, maybe due to the more sensitive *in situ* hybridization technique. This information allows us to speculate that the compensatory and possibly additive or competitive mechanisms of Arnt and Arnt2 are wider, especially in the peripheral organs (excluding the liver, heart and brown fat) than what has been expected according to the earlier studies.

3. IMPLICATIONS FOR A ROLE FOR Aint/Tacc3 IN CELL PROLIFERATION IN DEVELOPING MURINE TISSUES AND IN SPERMATOGENESIS AND OOGENESIS

As a result of the search for novel Arnt interacting proteins, Aint was identified. The same protein was concurrently discovered and named Tacc3 by Still and the co-workers (1999b). It has also been found by two other independent groups as a result of the search for erythopoietin induced genes in erythroid progenitors (McKeveney et al. 2001) and Stat5-interacting gene products (Piekorz et al. 2002). We also showed that Aint interacts strongly with Arnt2 and minimally with Sim2 but not with other bHLH-PAS proteins studied. We decided to use term Tacc3 instead of Aint in order to avoid confusion caused by diverse nomenclature.

Tacc3 belongs to the family of transforming acidic coiled coil proteins. The analysis of the coiled coil structure suggests that the TACC proteins could form multiprotein complexes. Yeast two-hybrid assays with fragments of Tacc3 indicate that Tacc3 could be interacting with the PAS domain of Arnt, possibly in the same way as the non-bHLH-PAS circadian clock protein Timeless (TIM) interacts with the PAS domain of *Drosophila* Period (PER) (Gekakis et al. 1995, Sehgal et al. 1995). *In vitro* also TACC1 has been shown to be capable of interacting with two tumor associated proteins (Lauffart et al. 2002) supporting the hypothesis that Tacc3 might form multiprotein complexes *in vivo*.

The subcellular localization of Tacc3 in our immunocytochemial analyses was solely nuclear. Other groups have also shown cytoplasmic localization. As can be predicted from the protein sequence, Tacc3 has been observed in the centrosomal region and mitotic spindle in mitotic cells (Piekorz et al. 2002). In interphase cells Tacc3 has been localized to

cytoplasm and perinuclear region (Piekorz et al. 2002). Also human TACC3 has been found in centrosomes of mitotic cells whereas it was diffusely distributed in the cytoplasm and nucleus in interphase cultured HeLa cells (Gergely et al. 2000b). Similarly, human TACC1 and TACC2 have been proposed to play a role in organizing centrosomal microtubules (Gergely et al. 2000b).

In *Drosophila,* a related protein D-TACC has a role with mini-spindels protein (Msps), the homolog of the human colonic and hepatic tumor overexpressed protein (ch-TOG), in the spindle function in the early embryo (Gergely et al. 2000a, Cullen and Ohkura 2001, Lee et al. 2001). It has been proposed that this interaction plays a role in the stabilization of centrosomal microtubules (Cullen and Ohkura 2001, Lee et al. 2001). The function of mammalian TACC proteins in cytoplasm might be to function in the organization of the microtubule network as scaffolding/bridging proteins.

In addition to centrosomal assistance, Tacc3 might have also a nuclear role. It is *in vitro* capable of binding with GAS41/NuBI1-containing basic transcription factor (Lauffart et al. 2002). Linking together this and our results demonstrating nuclear localization of Tacc3, upregulation of ARNT-mediated responses to hypoxia and chemical inducers allows the speculation that Tacc3 could have a role as a linking protein between transcription factors (such as ARNT) and basal transcription initiation complexes.

Human *TACC* genes have been observed to be located in genomic regions that are rearranged in certain cancer cells. *TACC3* is upregulated in several tumor cell lines (Still et al. 1999b). *TACC1* was cloned from breast cancer amplicon and its constitutive expression in mouse fibroblasts resulted in cellular transformation (Still et al. 1999a). TACC2/AZU-1 is a candidate breast tumor suppressor (Chen et al. 2000). *Xenopus* maskin is involved in regulating the translation of specific mRNAs in the developing frog embryo (Stebbins-Boaz et al. 1999). The association with tumorigenesis may be due to the disturbances in transcriptional regulation by TACC proteins.

The *in vitro* interaction partners of Tacc3, Arnt and Arnt2, have important roles in murine development as demonstrated by gene targeting experiments (Kozak et al. 1997, Maltepe et al. 1997, Hosoya et al. 2001 and Keith et al. 2001). Also the hypoxia response pathway, where Tacc3 is thought to be involved, is crucial for mammalian development (Iyer et al. 1998, Ryan et al. 1998, Kotch et al. 1999, Semenza et al. 1999). Our results of *Tacc3* mRNA expression and Tacc3 immunoreactivity during embryonic and postnatal murine development propose a role for Tacc3 in mouse development. The expression of *Tacc3* mRNA was seen in proliferating cells both in developing central nervous system and in peripheral organs during the period of most active cell division (E13 to P1.5) and subsequently the downregulation of *Tacc3* was observed.

The importance of Tacc3 for murine development has been confirmed by analysis of Tacc3-deficient mice created by Piekorz et al. (2002). The $Tacc3^{-/-}$ embryos died at mid- to late gestation and striking growth retardation was detected. Cell proliferation was studied especially in hematopoietic stem cells and the main reason for unsuccessful cell proliferation was observed to be in increased apoptosis and high levels of p53 target protein

$p21^{waf1/cip1}$ were seen in the livers of $Tacc3^{-/-}$ mice. The $p53^{+/-}$/$Tacc3^{-/-}$ double knockout mice were viable - demonstrating that the lack of Tacc3 protein triggers p53-mediated apoptosis which could be prevented by p53-deficiency. The consequences of Tacc3 deficiency in other cell lines than hematopoietic cells were not studied (Piekorz et al. 2002). Accordingly, McKeveney et al. (2001) have shown that ERIC-1/TACC3 is upregulated by erythropoietin (Epo) and have a role in terminal erythropoiesis.

Interestingly, in the testis we saw low *Tacc3* mRNA levels during pre-natal and early postnatal development, whereas expression increased in puberty and in the adult. The cells with high expression were primary and secondary spermatocytes, which are going through first and second meiotic divisions. These findings suggest a role for Tacc3 in mammalian spermatogenesis. The high expression in postnatal and adult ovary proposes a role also in oogenesis.

Localization of Tacc3-immunoreactivity, which was studied during the embryonic period and at P2 was mostly congruent with that of Tacc3 mRNA seen by *in situ* hybridization. However, some discrepancies were seen. For example, in the central nervous system, a weak immunoreactivity was observed also in the neurons of differentiating fields, although mRNA levels were undetectable. This may be due to the differences in sensitivity of the methods. In the liver, a weak immunoreactivity in hepatocytes continued postnatally, whereas no mRNA was observed any more at E17. In the pancreas, immunoreactivity was seen from E13 onwards. With *in situ* hybridization method we failed to examine this organ because of the problem caused by endogenous RNAases. In adult testes, the *Tacc3* transcripts were seen at high levels only in primary and secondary spermatocytes although weaker levels were detected in early round spermatides. Instead, the immunoreactivity was observed more widely in P2 mouse (Sertoli cells, primitive germ cells, interstitial cells, peritubular cells and fibroblasts of tunica albuginea). Immunohistochemical analysis by McKeveney et al. (2001) revealed hERIC3/TACC3 localization in the cytoplasm of Sertoli cells of adult human testis, which is contradictory to our findings showing Tacc3-IR only in spermatogenic cells of adult human testis (Sadek et al., unpublished results).

The expression of *Tacc3* in rapidly proliferating mouse tissues suggests a crucial role in cell proliferation during mouse development. This is supported by studies of other members of TACC family and their roles in organizing centrosomal microtubules as well as connections with tumor formation. In addition, the high levels of Tacc3 in testis and ovary, proposes a role in spermatogenesis and oogenesis. Whether the function of Tacc3 is to contribute to the hypoxia response or aryl hydrocarbon receptor pathways or is associated with other mechanisms, remains to be clarified by further studies.

SUMMARY AND CONCLUSIONS

This thesis focused on three fox proteins (Foxf1, Foxf2 and Foxe3), two bHLH-PAS proteins (Arnt and Arnt2) and a novel Arnt interacting protein Aint/Tacc3 in association with murine development.

Foxf1 and Foxf2 were previously known to function as transcriptional activators and their developmental expression was now mapped in mouse tissues. Here, the transcriptional activation properties of FOXF1 were examined more closely. Two differently functioning activation domains were determined, one cell-type-specific located to the central part of the protein and another general activation domain in C-terminal part of the protein. The human *FOXF1*gene was characterized and shown to consist of two exons separated by an intron of 1.2 kb. Exon 1 encodes the DNA binding domain and the cell-type-specific domain whereas exon 2 encodes the general activation domain.

The expression of *Foxf1* and *Foxf2* was studied during embryonic development by *in situ* hybridization in mouse and rat tissues. The investigation of *Foxf2* expression was continued postnatally. *Foxf1* and *Foxf2* exhibit largely overlapping expression patterns, although both qualitative and quantitative differences were found. The main sites of expression for both *Foxf1* and *Foxf2* were the respiratory, alimentary and urinary tracts, where the transcripts were localized to the mesenchyme adjacent to the epithelium. This type of expression pattern suggests that these factors may have similar functions in epithelio-mesenchymal crosstalk, which is crucial for organ development. In the unparalleled expression sites *Foxf2* may have an independent role as a developmental regulator. The expression of *Foxf2* continued until adulthood in the eye, stomach, intestines and lung.

A mouse homolog of *FOXE3* was cloned and sequenced and its role examined in the developing mouse. Strong evidence was found that Foxe3 is essential for closure of the lens vesicle and lens epithelial proliferation. Its expression in the mouse embryo was limited to the lens of the developing eye and, during a short period, to a small site on the neural folds in the cephalic region. Expression in the lens was first observed at the start of the lens placode induction around E9.5. Later, the expression in the lens became restricted to the anterior proliferating cells at the time when lens differentiation begins. Previously, the classic mouse mutant *dysgenetic lens* has been described where the lens vesicle fails to separate from the ectoderm leading to fusion between lens and cornea (Sanyal and Hawkins 1979). In *dyl* mice, instead of the proliferation characteristic for a normal lens epithelium, the posterior of these cells fail to divide and show signs of premature differentiations, whereas the most anterior cells are eliminated by apoptosis. The chromosomal location of mouse *Foxe3* was found to colocalize with that of *dyl* mutation. The entire coding region of *Foxe3* was sequenced from a homozygous *dyl* mouse, and mutations were found in the part of *Foxe3* encoding the DNA-binding domain. These mutations cosegregate with the *dyl* phenotype. Our results suggest that mutations in *Foxe3* are responsible for the *dyl* phenotype. Evidence was provided that the role of Foxe3 in the lens epithelial cells might be to promote survival and proliferation, while preventing differentiation.

bHLH-PAS proteins Arnt and Arnt2 affect transcriptional regulation by functioning as dimerizing partners for several related, and also some non-related, partners. We provided a detailed description of the expression of *Arnt* and *Arnt2* mRNA in mouse tissues during embryonic and early postnatal development by *in situ* hybridization. The expression of both *Arnt* and *Arnt2* was wide, but showed a temporally and spatially specific pattern. In the developing central nervous system, the expression of *Arnt* and *Arnt2* was almost equal, although the expression of *Arnt2* continued temporally further. Several novel sites of expression for *Arnt* and especially for *Arnt2* were observed. Their expression was parallel in many tissues where they may compete or substitute for common heterodimerizing partners.

In the search for novel Arnt interacting proteins, Tacc3 was cloned and sequenced. In addition to Arnt, interaction with Arnt2 and minimally with Sim2 were observed. The C-terminus of Tacc3 was shown to mediate the interaction with the PAS domain of Arnt in yeast. Our immunocytochemical results showed nuclear localization of Tacc3. Tacc3 overexpression was shown to facilitate signaling via the hypoxia and xenobiotic response elements. A high and widespread expression of *Tacc3* was seen during fetal development from E13 onwards until 1.5 days after birth. The expression in the central nervous system and in the peripheral tissues was associated with the most active proliferation period, implying that Tacc3 is needed for cell proliferation. The simultaneous expression of *Tacc3* with its *in vitro* interaction partners *Arnt* and *Arnt2* was observed in several, but not all, sites.

In some organs, expression continued postnatally; however, in the adult mouse high amounts of *Tacc3* mRNA were present only in the testis and ovary, and lower levels were detected in the thymus and spleen. Expression in the testis was low during pre-natal and early post-natal development. However, when the mice matured and became fertile *Tacc3* mRNA levels increased remarkably, suggesting that Tacc3 has an important role in mammalian spermatogenesis. In the ovary, the high expression of *Tacc3* in oocytes implies that it has a role in oogenesis.

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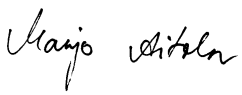

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