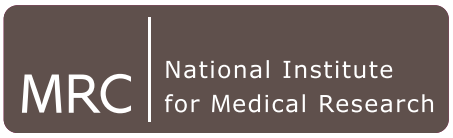

FOXL2 represses the testis-specific enhancer of *Sox9* to maintain ovary differentiation

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Für meine Eltern

I, Susanne Jakob, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Susanne Jakob

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Δ	floxed out or deleted allelle
°C	degree Celsius
μg	microgram
μl	microlitre
μM	micromolar
μm	micrometre
bp	base pair
BPB	bromphenol blue
BSA	bovine serum albumin
cDNA	complementary DNA
CFP	cyan fluorescent protein
Ci	Curie
CMV	cytomegalovirus
cpm	counts per minute
Cre	Cre recombinase
DAPI	di-amino-phenyl-indol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpc	day(s) *post coitum*
dpp	day(s) *post partum*

DTA	diphtheria toxin fragment A
DTT	dithiothreitol
ECFP	enhanced cyan fluorescent protein
EDTA	ethylen diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylen glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERT	estrogen receptor for tamoxifen
ESD	environmental sex determination
EYFP	enhanced yellow fluorescent protein
FCS	fetal calf serum
flox	flanked by loxP sites
GFP	green fluorescent protein
GoF	gain of function
GSD	genetic sex determination
GST	glutathion-S-transferase
H+E	haematoxylin-eosin
HA	hemagglutinin epitope tag
HEPES	4-2-hydorxyethyl-1-piperazineethanesulfonic acid
HMG	high mobility group
hsp	heat shock protein
IgG	immunglobulin G
IP	immunoprecipitation
kb	kilo base
LacZ	β-galactisodase gene
LB	Luria Broth
LoF	loss of function
loxP	locus of cross-over P
M	molar

min	minute
NDS	normal donkey serum
neo	neomycin gene
NES	nuclear export signal
OCT	optimal cutting temperature
OD	optical density
OHT	4-hydroxytamoxifen
ONPG	O-Nitrophenyl-β-D-galactopyranosid
pA	polyadenylation
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformadehyde
PGC	primordial germ cell
PGK	phosphoglycerine kinase
PMSF	phenylmethylsulphonyl fluoride
PTM	peritubular myoid
rpm	revolution per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
ssc	side chain cleavage
TBE	tris borate EDTA
TES	testis specific enhancer of *Sox9*
TESCO	testis specific enhancer of *Sox9* core element
Tris	tris(hydroxylmethyl)aminomethane
TSD	temperature dependent sex determination
U	units
VP	vaginal plug
w/v	weight to volume

Abstract

During normal testis development, SRY and SF1 up-regulate *Sox9* expression via its testis-specific enhancer element (TESCO), which is crucial for the establishment of the male supporting cell lineage, the Sertoli cells. In contrast, during normal ovarian development, *Sox9* expression needs to be down-regulated in the female supporting cells and failure results in XX female-to-male sex reversal. It was not known whether the repression of *Sox9* expression is mediated via the TESCO regulatory element in XX gonads. FOXL2 is a forkhead transcription factor, expressed in the supporting cells of the ovary, but not testis, from 12.5 dpc onwards. Homozygous mutation of *Foxl2* results in the up-regulation of *Sox9* and other testis-specific genes in postnatal gonads, suggesting that FOXL2 plays an important role in proper ovarian development by maintaining the repression of *Sox9*. In this thesis, *in vitro* analyses demonstrate that FOXL2 can repress TESCO activity (as can other candidate ovarian determining genes, such as *Dax1* and *Sox4*). The repressing effect of FOXL2 is more severe in the presence of ERα. Several forkhead factor binding sites and EREs are present in the TESCO sequence, however mutation analyses showed that the repression effect is not mediated only through these. Moreover, FOXl2 can interact with SF1 and interfere with its ability to activate TESCO *in vitro*. *In vivo* studies showed that homozygous loss of *Foxl2* results in the de-repression of TESCO activity in the remaining granulosa-like cells in the XX gonads, which correlates with the expression of endogenous *Sox9*. However, this de-repression occurs only after birth, getting more severe with age, suggesting that *Foxl2* is the critical factor to re-

press TESCO activity in the adult ovary. Moreover, evidence is provided that the de-repression of *Sox9* is independent from oocyte-depletion and solely due to the loss of *Foxl2* as targeted deletion of oocytes did not result in an up-regulation of *Sox9* expression. Further *in vivo* analyses indicate the involvement of Wnt signalling in the repression of TESCO activity during embryonic development, as TESCO becomes partially de-repressed in *Wnt4* homozygous mutant mice before birth.

Chapter 1

Introduction

1.1 Mechanisms of sex determination

During evolution, the complex living organisms on earth have evolved through generations of sexual reproduction, whereas asexual organisms mostly remain primitive and simple. The benefit of sexual reproduction comprises genetic variation, leading to the uniqueness of each individual, which is fundamental to the success of the species.

The common precondition for sexual reproduction is the establishment of different sexes in one species. While in the majority of vertebrates all individuals are phenotypically identical at the beginning of embryogenesis, each embryo has to take the decision to develop into one sex, and not the other, at a specific time point during its development. This event is called sex determination. Sex determination happens in the gonads of the embryo, more precisely in the somatic cells of the gonad, and describes the decision of the originally bi-potential gonad to develop either into a testis or an ovary. Once this switch has been made, the

course is set for the rest of the embryo to develop either as a male or a female individual. However, many invertebrate species and some vertebrates, e.g. some fish species, develop as hermaphrodites which can produce both sperm and eggs either simultaneously or at successive life stages. In most species, sex determination is achieved during embryonic development, though there are examples of individuals, e.g. in some fish species, which are able change their sexual phenotype during adult life.

The triggers through which sex determination is achieved in different species are manifold. The switch mechanism can dependent on either environmental or genetic factors. Environmental sex determination (ESD) is based on the identical genetic makeup of all individuals and environmental events triggering distinct sexual phenotypes. In contrast, genetic sex determination (GSD) relies on the differences in chromosomal constitution (or even in a single gene) between individuals, that is the presence of distinct sex chromosomes.

A well studied form of ESD is temperature dependent sex determination (TSD) in which the temperature during a critical period of egg incubation determines the sex of the offspring, e.g. in crocodiles, alligators, most turtles and some lizards. For example, the red-eared slider turtle (*Trachemys scripta*) develops as male at an egg incubation temperature lower than 26°C and as female at temperatures higher than 31°C. Between 26°C and 31°C both sexes can develop with the percentage of females increasing with rising temperature (Pieau et al., 1999). Other ESD mechanisms have been described to depend on group dynamics including visual and hormonal signals, e.g. in Clown fish (*Amphiprion ocellaris*) (for review see Gilbert (2006)).

In contrast to ESD, GSD is determined by the genetic content or chromosomal constitution of each individual and has been found in, amongst others, worms, insects and most vertebrate species. In some species, the development of distinct sexual phenotypes depends on the presence of a haploid or a diploid genome (e.g. in bees and ants). In others, specific sex chromosomes have evolved leading to chromosomal differences between the sexes. Here, the sex determining switch can be controlled by a dosage-dependent mechanism (DSD) or the presence of a dominant gene on one of the sex chromosomes. DSD can be found in *C. elegans* and *D. melanogaster*, in which the sex determination switch is controlled by the ratio of X chromosomes to autosomes (although e.g. *Drosophila* has a Y chromosome which is required for male fertility but not for sex determination). Sex determining mechanisms which rely on sex-specific chromosomes have been described in the ZZ/ZW system (e.g. in some insects, fish, reptiles and birds) and the XX/XY system (e.g. in some fish, amphibians, reptiles and all known mammals). In the ZZ/ZW system, homogametic ZZ embryos develop as males whereas heterogametic ZW embryos develop as females. For a long time, two possible mechanisms of sex determination have been discussed in this system: a dosage-dependent effect of Z chromosome gene(s) and the activity of female-specific gene(s) from the W chromosome. Recently, it has been shown in chick that the Z-linked gene *Dmrt1* is essential for male sex determination (Smith et al., 2009) supporting the Z-dosage dependent hypothesis. However, it is still not known whether *Dmrt1* is sufficient to induce male development. Furthermore, it is also possible that the role of *Dmrt1* is to antagonise crucial gene(s) on the W chromosome which might be necessary for female development. In the mammalian XX/XY system, the Y

chromosome is the dominant male determinant and males are heterogametic XY whereas females are homogametic XX. The first hints for the importance of the Y chromosome for mammalian sex determination came from studies in human patients with Turner (45, X) and Klinefelter (47, XXY) syndrome, where XO individuals develop as females and XXY individuals develop as males (Ford et al., 1959; Jacobs and Strong, 1959). The same correlations were found in studies in mice (Welshons and Russell, 1959). These findings led to the proposal of the Y chromosome as the male determinant in mice and men. The subsequent quest for the specific testis determining factor (TDF) on the Y chromosome went on for 30 more years. Finally, in 1990, the analysis of several XX male patients led to the discovery of a conserved 35 kb region on the Y chromosome. Within this region, the male determining gene was identified by positional cloning and named *SRY* for "sex-determining region of the Y chromosome" (Sinclair et al., 1990; Gubbay et al., 1990).

1.2 Overview of murine gonadogenesis

In mice, SRY stands at the top of a sex determination cascade, being the switch between female and male development. Before going into detail about how the presence or absence of SRY triggers the molecular events that will eventually result in the formation of either a testis or an ovary, a closer look should be taken at the morphology of the embryonic gonad as the place were primary sex determination takes place. The morphological development of murine gonads can be divided into two phases: the development of a bi-potential gonad which is

morphological identical in both sexes and the subsequent sex-specific differentiation into either a testis or an ovary following the time point of sex determination around 11.5 dpc (days *post coitum*).

In the early embryo, the undifferentiated genital ridge can be recognised as a thickening of the coelomic epithelium adjacent to the mesonephros around 10.0 dpc. Shortly afterwards, primordial germ cells (PGCs) start to colonise the gonads (Figure 1.1). PGCs emerge as a cluster in the region posterior to the primitive streak at 7.25 dpc and then proliferate and migrate through the gut mesentery to enter the genital ridge between 10 and 11 dpc (Ginsburg et al., 1990). PGCs are indifferent between the sexes when they enter the genital ridge, and the special environment of the gonad is needed for adequate differentiation into either spermatogonia or oogonia (Saitou et al., 2002). In the male, germ cells then enter mitotic arrest in G0/G1 around 13.5 dpc until perinatal stage, whereas in the female they enter meiotic prophase as oocytes around 13.5 dpc and will then arrest in diplotene phase around the time of birth (McLaren, 1984).

While the germ cells originate outside of the genital ridge, the somatic cells of the gonad are thought to arise from proliferation of the coelomic epithelium. The somatic cells in the developing gonad are categorised into three major cell lineages based on their cell fate after sex determination. Supporting cell precursors develop into Sertoli cells in the male and follicle (granulosa) cells in the female. This cell lineage was named after their function to support and maintain the germ cells and is most crucial for gonad differentiation in both sexes. Steroidogenic cell precursors develop into Leydig cells in the male and theca cells in the female.

Finally, the connective cell lineages which are involved in the formation of the organ as a whole show distinct testicular and ovarian patterns of differentiation.

At the time of sex determination in XY gonads, *Sry* is expressed exclusively in supporting cell precursors, which originate from the coelomic epithelium, and directs their fate to become Sertoli cells (Koopman et al., 1990; Karl and Capel, 1998). Directly after initiation of *Sry* expression, proliferation of coelomic epithelial cells increases, a phenomenon specific to XY gonads (Schmahl et al., 2000). During this male-specific proliferation, cells from the epithelium delaminate and give rise to Sertoli cells and an interstitial cell population (Karl and Capel, 1998; Schmahl et al., 2000). The differentiating Sertoli cells then enclose the germ cells and form testis cords. These eventually give rise to the seminiferous tubules characteristic of the testis (Figure 1.1) (Brennan et al., 2002).

Leydig cells differentiate in the interstitium between the testis cords around 12.5 dpc. The function of Leydig cells is to produce testosterone and thereby guide the masculinisation of the whole embryo. In contrast to Sertoli cells, the origin of fetal Leydig cells is not clear yet. Some evidence suggests that Leydig cell precursors could arise from the coelomic epithelium (Brennan et al., 2003), whereas other data suggests that Leydig precursor cells originate from the mesonephros and migrate into the gonad before 11.5 dpc where they remain undifferentiated until 12.5 dpc (Jeays-Ward et al., 2003). As cell division is rarely detected among differentiated Leydig cells, it has been proposed that the increase in the fetal Leydig cell population depends on an undifferentiated pool of progenitor cells in the gonadal interstitium which continuously proliferates and differentiates (Orth, 1982). Recent studies strongly support this model

and show that Notch signalling is involved in the regulation of the maintenance of these fetal Leydig progenitor cells (Tang et al., 2008). Furthermore, signalling molecules from Sertoli cells have been implicated to be involved in the differentiation of fetal Leydig cells, such as *Dhh* (Desert hedgehog) and PDGF signalling (Platelet-Derived Growth Factor) (Yao et al., 2002; Brennan et al., 2003; Schmahl et al., 2008). The fetal Leydig cell population is lost at birth and replaced by an adult population which forms from interstitial mesenchymal cells during puberty (Davidoff et al., 2004). It has been hypothesised that the adult Leydig cells might differentiate from the same Leydig cell progenitor cell pool as the fetal Leydig cells (Habert et al., 2001; Tang et al., 2008).

In the XY gonad, testis-specific peritubular myoid (PTM) cells can be found, which surround the testis cords and thereby separate the cords from the interstitium (Figure 1.1). Originally these cells were thought to emerge from the mesonephros (Merchant-Larios and Moreno-Mendoza, 1998), but recent studies have shown that the PTM cells do not migrate from the mesonephros into the gonad between 11.5 dpc and 12.5 dpc (Cool et al., 2008; Combes et al., 2009). It has then been hypothesised that PTM cells and other interstitial lineages (except endothelial cells) are induced within the gonad.

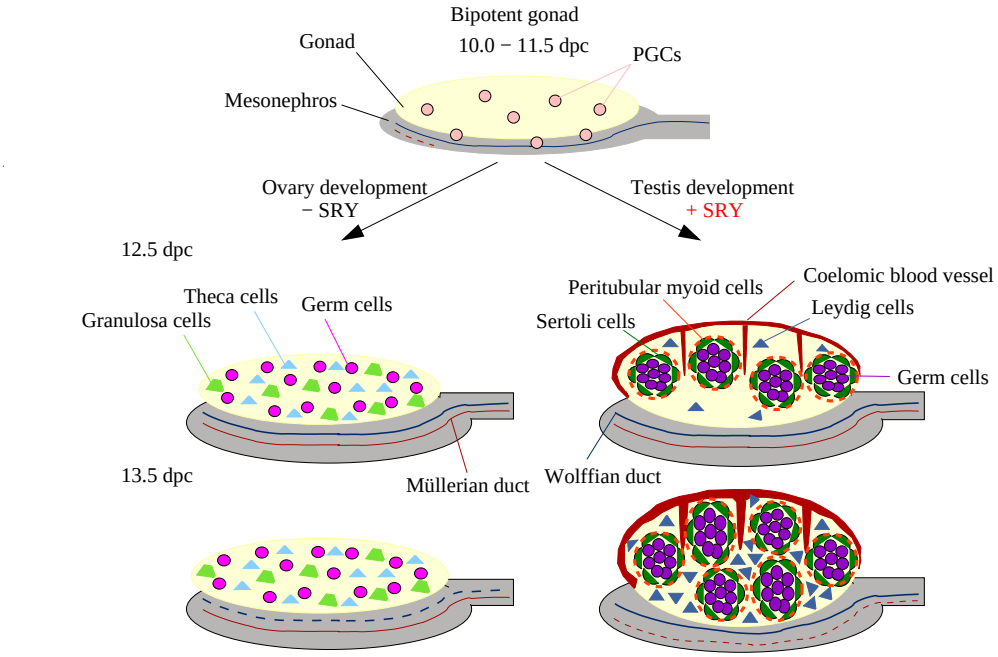
Endothelial cells start migrating into the XY gonad from a large vascular plexus in the mesonephros around 11.5 dpc (Coveney et al., 2008). These individual cells migrate directly towards the coelomic epithelial domain of the gonad where they aggregate and from the male-specific coelomic vessel around 12.5 dpc. The migrating endothelial cells have been suggested to direct the formation of the developing testis cords (Combes et al., 2009). It has also been suggested that

testicular blood vessels promote efficient export of testosterone out of the developing testis, as well as supplying oxygen to the rapidly growing tissue (Ross and Capel, 2005).

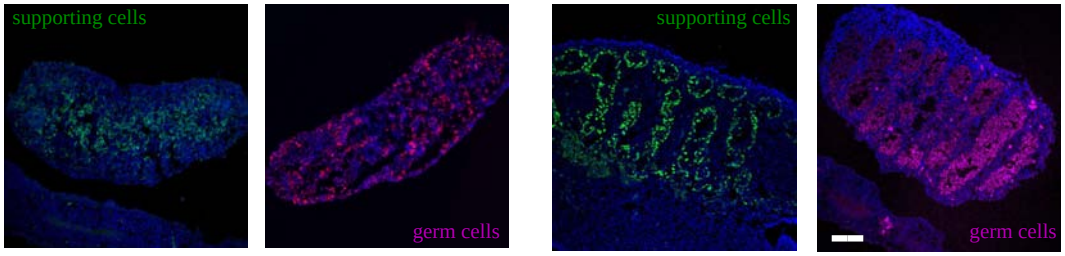
In the XX gonad, supporting cell precursors differentiate into follicle (granulosa) cells whereas the steroidogenic cell lineage gives rise to theca cells (Figure 1.1). Both cell types are involved in the production of estrogens. Although endothelial cells migrate from the mesonephric vascular plexus into the gonad from 11.5 dpc, no vascular pattern is obvious in the XX gonad at 12.5 dpc (Coveney et al., 2008). It has been suggested that the developing vasculature in XX gonads results from proliferation and extension of the primordial gonadal vasculature (Coveney et al., 2008). In the ovary, most of the morphological reorganisation occurs after birth. However, some morphological changes already start at the time of the entry of the germ cells into meiotic prophase around 13.5 dpc. At this time, oocytes become associated in clusters which are called germ cell nests or cysts (Gomperts et al., 1994; Pepling and Spradling, 2001). Each cluster of oocytes is surrounded by a layer of pre-granulosa cells. The cysts break down around the time of birth and while approximately two-third of the oocytes undergo programmed cell death, the remainder individually become surrounded by somatic pre-granulosa cells and form primordial follicles (Pepling and Spradling, 2001).

The internal reproductive tract and associated organs are derived from a pair of genital ducts, the Müllerian and Wolffian ducts, which are initially present in both sexes (Figure 1.1). The Wolffian ducts develop from the intermediate mesoderm and are completed by 10.5 dpc. In XY mice, they differentiate into

vas deferens, epididymis and seminal vesicles, whereas they regress in XX. The Müllerian ducts start to develop around 11.5 dpc. They give rise to oviduct, uterus and the upper parts of the vagina in XX mice, whilst they degenerate in XY due to the presence of anti-Müllerian hormone (AMH) made by Sertoli cells. It has been reported that the Müllerian duct needs the presence of the Wolffian duct to form (Gruenwald, 1941). However, the Wolffian duct does not contribute cells to the Müllerian duct, but is thought to provide essential (though still unknown) signals to guide its development (Orvis and Behringer, 2007).



(a)



(b)

Figure 1.1: **The early development of testis and ovary.**
(a) The genital ridge forms as a thickening of the coelomic epithelium around 10.0 dpc and primordial germ cells (pink) enter between 10.0 and 11.0 dpc. Both, the Müllerian and the Wolffian duct form initially in the mesonephros underlying the bi-potential gonad. In XY gonads, *Sry* is expressed between 10.75 and 12.5 dpc and initiates testis development: differentiation of supporting cell precursors to Sertoli cells (green) which, together with the peritubular myoid cells (orange), encircle the germ cells (purple) to form testis cords. In between the testis cords develop the male steroidogenic cell lineage: the Leydig cells (blue). The male-specific vasculature (red) forms and vessels branch between the testis cords. In contrast, no significant morphological changes are visible in XX gonads during this period. Adapted from Ross and Capel (2005). (b) The distribution of germ cells (purple, marked by STELLA) and supporting cells (green, marked by FOXL2 in XX gonads and SOX9 in XY gonads) in the embryonic gonad at 13.5 dpc. Scale bar = 100μm

1.3 Early gonadal development

In the indifferent gonad, several genes are expressed in both sexes from 9.5 dpc onwards, including Wilms' tumour suppressor gene 1 (*Wt1*), steroidogenic factor 1 (*Sf1*), LIM homeodomain protein 9 (*Lhx9*), M33 and *Emx2*. In mice, functional mutations of these genes cause gonadal agenesis, indicating that they are required for the early formation of the bi-potential gonad prior to the time of sex determination.

Wt1 was originally identified as a gene involved in infant kidney cancer and mutations and deletions of the gene are associated with WAGR (Wilms' tumour/aniridia/genitourinary abnormalities/mental retardation), DDS (Denys-Dash syndrome) and FS (Frasier syndrome). The gene is composed of ten exons, which undergo alternative splicing and show different translational start sites, resulting in a total of 24 different protein isoforms (Reddy and Licht, 1996; Sharma et al., 1994), which share a characteristic domain of four zinc fingers in the C-terminus. Of particular interest for gonadal development are two splice variants which differ in the insertion or omission of the three amino acids KTS between the third and fourth zinc finger (+KTS and -KTS isoforms). The two protein isoforms vary in their DNA binding activity *in vitro* (Laity et al., 2000) and only the +KTS isoform is able to bind and stabilise RNA (Bor et al., 2006). Unlike human patients, mice with a heterozygous null mutation of *Wt1* do not show any gonadal phenotype. Mice with homozygous *Wt1* null mutations die between 13 dpc and 15 dpc, due to a failure of kidney development, and lack both gonads and adrenal glands (Kreidberg et al., 1993). Targeted deletion of either the

+KTS or -KTS isoforms revealed their distinct function during gonadal development. While WT1(+KTS) seems to be more important in testis development, WT1(-KTS) is required for formation and survival of the gonadal primordium in both sexes, as WT1(-KTS$^{\Delta/\Delta}$) mice show an increased number of apoptotic cells in the early gonad (Hammes et al., 2001).

Steroidogenic factor 1 (*Sf1*, *Nr5a1*) is a member of the nuclear receptor family and was first identified as an activator of genes involved in steroidogenesis (Parker and Schimmer, 1997). SF1 contains a DNA-binding domain composed of highly conserved zinc fingers and recognises (C/T)CAAGG(T/C)(C/T) and (A/G)(A/G)(A/G)AGGTCA DNA sequences (Morohashi et al., 1992). In human patients, heterozygous mutation of *SF1* is associated with primary adrenal failure, XY male-to-female sex reversal including different degrees of gonadal dysgenesis and persistent Müllerian structures (for review see Lin and Achermann, 2008). In contrast to humans, mice with a heterozygous mutation of *Sf1* do not display any gonadal phenotype (Luo et al., 1994). Mice carrying a homozygous null mutation of *Sf1* (*Sf1*$^{-/-}$) start to develop the genital ridge correctly and migration of PGCs appears normal. However, somatic cells undergo apoptosis around 11 - 11.5 dpc, which results in the complete lack of gonads and adrenals, indicating an essential role for *Sf1* in regulating the survival and proliferation of the adrenal and somatic gonadal cells (Luo et al., 1994).

Lhx9 is a member of the LIM homeobox transcription factor family with two N-terminal LIM domains and a homeobox domain. The LIM domain is a cysteine-rich domain consisting of two zinc fingers which mediates protein-protein inter-

actions, whereas the homeobox domain is involved in DNA binding. Mice with a homozygous null mutation of *Lhx9* (*Lhx9*$^{-/-}$) show failure of somatic cell proliferation in the genital ridge, resulting in the absence of gonads (Birk et al., 2000). *Sf1* expression is significantly reduced in *Lhx9*$^{-/-}$ mice, indicating that LHX9 modulates *Sf1* activation. Indeed, it has been shown *in vitro* that LHX9 and WT1(-KTS) interact and cooperatively activate the *Sf1* promoter (Wilhelm and Englert, 2002).

The *M33* gene (also known as *Cbx2*) is a member of the *polycomb* (*PC*) group of proteins which were first identified in *Drosophila melanogaster*. Mice with homozygous deletion of the M33 C-terminus (*M33*$^{cterm/cterm}$), which is highly conserved among *Drosophila Pc* proteins (Pearce et al., 1992), show retardation in the formation of the genital ridge in both sexes (Katoh-Fukui et al., 1998). In adult XX *M33*$^{cterm/cterm}$ mice, ovaries are either small or residual, whereas XY *M33*$^{cterm/cterm}$ mice show varying degrees of male-to-female sex reversal with the appearance of ovarian-like structures containing follicles. Further analyses of the *M33* mutant mice indicated a reduced expression of *Sf1* (Katoh-Fukui et al., 1998). Moreover, M33 directly binds to the *Sf1* gene locus *in vitro*, suggesting that M33 may be involved in the regulation of *Sf1* expression (Katoh-Fukui et al., 2005). Recently mutations in *CBX2* were identified in a human patient with XY male-to-female sex reversal (Biason-Lauber et al., 2009).

Emx2 is a homeobox gene which was first identified due to its homology to the *Drosophila* gene *empty spiracles* (Simeone et al., 1992). Mice carrying a homozygous null mutation of *Emx2* (*Emx2*$^{-/-}$) show a complete absence of gonads due

to a failure in thickening of the coelomic epithelium at 11.5 dpc. Moreover, the genital tract system is absent in both sexes in the *Emx2*$^{-/-}$ mice as the Müllerian ducts never form and the Wolffian ducts start to degenerate around 11.5 dpc in XY *Emx2*$^{-/-}$ mice (Miyamoto et al., 1997).

In summary, before 11.0 dpc the gonad is indistinguishable between XY and XX embryos. In both sexes, the same set of regulatory genes is expressed setting the stage for the changes to come and the gonad bears the potential to differentiate into either a testis or an ovary. And then along comes *Sry* and activates a cascade of gene activity which will eventually lead to the establishment of testis development in XY gonads.

1.4 Taking the route towards testes

1.4.1 Testis determination: *Sry*

SRY was shown to be critical for male sex determination through both loss- and gain-of-function studies. Analysis of human XY sex-reversed female patients discovered a *de novo* frameshift mutation in the Y-linked gene *SRY*, which indicated that it was necessary for testis development (Jäger et al., 1990). Subsequently, many XY females have been found with point mutations in *Sry* (for review see Sekido, 2010). Parallel studies confirmed the importance of *Sry* for testis determination in mice. Loss-of-function (LoF) experiments demonstrated that XY mice with a 11 kb deletion of *Sry*, which results in a lack of a functional *Sry* gene, develop ovaries and become female (Gubbay et al., 1990). Gain-of-function

(GoF) studies showed that XX mice carrying a *Sry* transgene develop testes and become male, although they are infertile (Koopman et al., 1991). These data demonstrate that *Sry* is both sufficient and necessary for testis development and therefore is only gene on the Y chromosome required for male development.

Studies in chimeric mice demonstrated that more than 90% of Sertoli cells in the testes were XY, in contrast to other cells which did not show any bias, suggesting that *Sry* acts in the male supporting cell lineage (Palmer and Burgoyne, 1991b). However, some of the cells were XX indicating that a non-cell autonomous factor can recruit XX cells to become Sertoli cells. By variation of the XX:XY cell ratio in the chimeric mice, a threshold level of 20% *Sry*-expressing cells was found to be sufficient for testis development (Burgoyne and Thornhill, 1993). Subsequent studies confirmed that *Sry* expression is restricted to cells that differentiate into Sertoli cells (Sekido et al., 2004; Wilhelm et al., 2005). These data substantiate the idea that the critical role for SRY in sex determination is to establish the Sertoli cell fate decision in the supporting cell lineage in the early gonad.

In the mouse, *Sry* is expressed in the Sertoli cell precursors of the XY gonad in a centre to pole wave (Bullejos and Koopman, 2004; Sekido et al., 2004; Albrecht and Eicher, 2001). Both mRNA and protein analyses showed that *Sry* is only present during a narrow time window between 10.75 dpc and 12.5 dpc in the nucleus of Sertoli cell precursors (Koopman et al., 1990; Sekido et al., 2004). *Sry* expression needs to reach a critical threshold per cell and also in a sufficient number of cells in order to initiate and maintain Sertoli cell differentiation (Palmer and Burgoyne, 1991a; Burgoyne and Thornhill, 1993). Recently, *ex vivo* studies

showed that XX gonads carrying an inducible *Sry* transgene only initiate correct Sertoli cell differentiation and thus testis development when the transgene was expressed in a 6 hour time window between 11.0 dpc and 11.25 dpc (Hiramatsu et al., 2009). These data show that if *Sry* is expressed at a critical time during gonadal development, it acts as a genetic switch to shift the development of the bi-potential gonad towards to male pathway.

Several factors have been proposed to be involved in the activation of *Sry* expression in the XY gonad, including SF1, WT1(+KTS), GATA4/FOG2, M33 and members of the insulin receptor (IR) family (Figure 1.2). Cell fate mapping analyses showing that *Sry*-expressing Sertoli precursor cells indeed originate from *Sf1*-expressing coelomic epithelial cells, suggests the importance of SF1 in *Sry* regulation and Sertoli cell differentiation (Schmahl et al., 2000). In support of this hypothesis, it has been shown that SF1 binds and activates both human and pig *Sry* promoters *in vitro* (de Santa Barbara et al., 2001; Pilon et al., 2003) and *Sry* expression can not be detected in gonads of XY $Sf1^{-/-}$ mice (S. Guioli, unpublished). FS patients lacking the WT1(+KTS) isoform also show XY sex reversal (Gubler et al., 1999). Recently, it has been shown that WT1(+KTS) is involved in the cell-autonomous regulation of *Sry* expression as gonads of XY ($+KTS^{\Delta/\Delta}$) mice show reduced *Sry* expression per cell as well as reduced proliferation of somatic cells and failure of Sertoli cell differentiation (Bradford et al., 2009). Mice carrying a homozygous null mutation of the zinc finger transcription factor *Gata4* ($Gata4^{-/-}$) die before 10.5 dpc due to severe defects in heart development (Molkentin et al., 1997). However, a hypomorphic mutation of *Gata4* ($Gata^{ki}$) which abolishes the interaction between GATA4 and its co-factor FOG2, or the

null mutation of *Fog2* (*Fog2*$^{-/-}$), both resulting in embryonic lethality after the time of sex determination, cause XY male-to-female sex reversal (Tevosian et al., 2002). *Sry* transcript levels are significantly decreased in XY *Fog2*$^{-/-}$ gonads (Tevosian et al., 2002). Recent studies revealed that GATA4 interacts with both WT1 isoforms (+KTS and -KTS) to activate the mouse, human and pig *Sry* promoters *in vitro* (Miyamoto et al., 2008). Combined triple deletions of IR genes, including IR itself, insulin-related receptor (IRR) and insulin-like growth factor 1 receptor (IGF1R), also result in a reduction of the *Sry* transcript level, associated with XY sex reversal (Nef et al., 2003). It is thought that this phenotype is due to a reduced proliferation of the somatic cells resulting in fewer *Sry*-expressing cells. However, an alteration of *Sry* transcription itself cannot be excluded. Recently, the involvement of MAPK (mitogen-activated protein kinase) signalling in the regulation of *Sry* expression has been described (Bogani et al., 2009). Loss of MAP3K4 activity results in XY sex reversal due to a failure in the up-regulation of *Sry* expression. However, it is not clear yet whether this failure of proper *Sry* expression is due to transcriptional regulation of *Sry* by the MAP3K4-signalling pathway or due to insufficient numbers of Sertoli cell precursors. Taken together, although a lot of genes have been implicated in *Sry* activation, little is still known about the exact transcriptional regulation of *Sry*.

Recent studies on the evolution of *Sry* have revealed that the 5' sequence upstream of *Sry* is partially conserved in human, pig, bovine and goat, including conserved binding sites for transcription factors, whereas the mouse sequence was found to be considerably different (Ross et al., 2008). In contrast to the transient expression of *Sry* in mouse, the expression of human and pig *Sry* persists

throughout later stages of development, which might suggest different regulatory mechanisms (Hanley et al., 2000; Daneau et al., 1996).

SRY proteins usually consist of three different regions: N-terminal domain, HMG box and C-terminal domain (Sekido, 2010). Interestingly, mouse SRY has a more unusual structure compared to other species. Due to a very short N-terminal domain, the HMG box is essential N-terminal. Moreover, the protein contains a large glutamine (Q) repeat region at the C-terminus which is connected to the HMG box by a bridge domain. *In vitro* SRY binds to the DNA consensus sequence (A/T)ACAA(T/A) via its HMG box and thereby bends DNA up to an angle of 90° (Ferrari et al., 1992; Giese et al., 1994). Outside the highly conserved HMG box domain, the conservation of the protein sequence is poor amongst mammals. The vast majority of point mutations in *SRY* in cases of human XY male-to-female sex reversal occur within the HMG box which suggests that this is the most critical part of the protein (Sekido, 2010). However, both the N- and C-terminal domains might play a role in *Sry* function, as point mutations in these regions have been found in a few cases of XY sex reversal (Shahid et al., 2004). The importance of the mouse-specific Q-stretch in male sex determination was suggested in transgenic mouse experiments, as *Sry* constructs lacking the Q-stretch were not able to induce testis development in XX embryos (Bowles et al., 1999). However, it was not possible to ascertain whether any mutant protein was actually made or stable in these mice. In contrast, the SRY proteins from *M. mus. domesticus* (Y^{DOM}) or *M. mus. poschiavinus* (Y^{POS}) contain a truncation of this Q-stretch. When Y^{DOM} or Y^{POS} chromosomes were transferred onto the *M. mus. musculus* background C57BL/6, ovarian tissue developed, indicating that these

Y chromosomes function as weak alleles, possibly due to the reduction of the *Sry* Q-stretch (Eicher et al., 1982). Recent studies indicated the involvement of the Q-stretch in interference with the female-promoting Wnt/β-catenin pathway (see later) (Tamashiro et al., 2008). In contrast to mouse SRY, human SRY seems to be unable to inhibit the Wnt/β-catenin, possibly due to the lack of the C-terminal Q-stretch domain. However, human SRY fused to the murine Q-stretch domain is able to interfere with this pathway (Tamashiro et al., 2008). Interestingly, even though human and mouse SRY are distinct in their C-terminus, hSRY expressed under the control of mouse regulatory sequences, can still induce testis development in the mouse (Lovell-Badge et al., 2002), as does the expression of goat *Sry* (Pannetier et al., 2006b).

Both human and mouse SRY is able to directly interact with other proteins, such as SRY-interacting protein (SIP-1) and Krüppel-associated box only protein (KRAB-O). SIP-1 was identified by an *in vitro* screen for human SRY interacting proteins (Poulat et al., 1997). Subsequent analyses revealed that SIP-1 interacts with the bridge domain between the HMG box and the Q-stretch of mouse SRY and with a specific motif (TKL-motif) at the C-terminus of human SRY (human SRY does not contain a bridge domain) (Thevenet et al., 2005). KRAB-O interacts with the bridge domains of mouse SRY and a domain adjacent to the C-terminus of the human SRY HMG box, but no clear motif has been identified (Oh et al., 2005). A recent study showed that ablation of *KRAB-O* expression *in vitro* via RNAi, results in a failure to up-regulate the SRY target gene *Sox9* (see later) (Polanco et al., 2009). However, targeted deletion of KRAB-O in mice did not impair testis development *in vivo*, which might be due to a possible

redundancy between members of the large KRAB gene family.

Interestingly, in the adult mouse testis, a circular *Sry* transcript has been found (Capel, 1998). This circular RNA is not translated and no function has been associated with it so far. It has not been found in humans or other mammals and might represent a mouse-specific mechanism to disable *Sry* function in the adult.

In summary, *Sry* must be expressed at the right time of development and in the right place in order to act as a molecular switch by activating downstream target genes which ensure that the supporting precursor cells become Sertoli cells and thereby setting the development of the bi-potential gonad towards testis development.

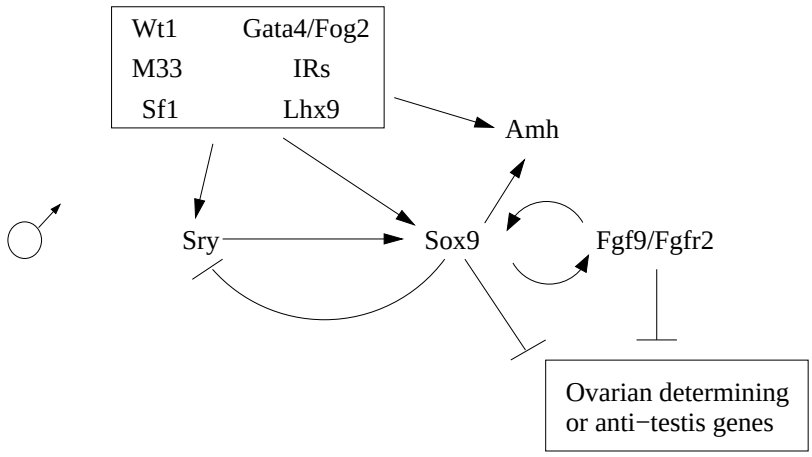


Figure 1.2: **Model of genetic interactions during testis development.** Possible genetic interactions in the XY gonad resulting in the establishment of *Sox9* expression in the supporting cell lineage. The testis-determining genes also have to ensure the inhibition of the ovarian-determining genes in order to maintain the Sertoli cell fate XY gonads.

1.4.2 Testis differentiation: establishment of Sertoli cells

As SRY acts only transiently in testis development, other genes, which act downstream of SRY, must be involved in the establishment of Sertoli cells. Several studies indicated the autosomal gene *Sox9* (Sex determining region Y, box 9) as the a crucial downstream target of SRY in testis development (Chaboissier et al., 2004; Sekido et al., 2004; Sekido and Lovell-Badge, 2008). *Sox9* is an *Sry*-related gene belonging to the Sox E family of HMG box proteins (together with Sox8 and Sox10). Like SRY, SOX9 can bind and bend DNA but it also contains a transactivation domain at the C-terminus, suggesting a role as a transcriptional activator (Bell et al., 1997; Lefebvre et al., 1997; Südbeck et al., 1996). In contrast to SRY which is found only in mammals, SOX9 is highly conserved amongst vertebrates.

Human patients with *SOX9* haploinsufficiency display a severe skeletal malformation syndrome, Campomelic dysplasia (CD), which is associated with XY male-to-female sex reversal in about 75% of all cases (Foster et al., 1994; Wagner et al., 1994). On the other hand, duplication of the chromosomal region containing *SOX9* (17q23-24) results in XX female-to-male sex reversal (Huang et al., 1999). In the mouse, homozygous null mutation of *Sox9* ($Sox9^{-/-}$) is embryonic lethal around the time of sex determination (Bi et al., 2001). However, when genital ridges from 11.5 dpc XY $Sox9^{-/-}$ mice were cultured *in vitro*, they neither formed testis cords nor expressed *Amh* (Chaboissier et al., 2004). Conditional deletion of *Sox9* via the Cre/LoxP system, using Sf1:Cre to delete *Sox9* specifically within the gonad ($Sox9^{\Delta/\Delta}$), can result in XY male-to-female sex reversal

(Chaboissier et al., 2004; Barrionuevo et al., 2006). Moreover, misexpression of *Sox9* in XX mice induces female-to-male sex reversal (Bishop et al., 2000; Vidal et al., 2001). These results mimic the LoF and GoF studies of *Sry*, respectively, implying that *Sox9* is the only critical downstream target of SRY and thereby is both necessary and sufficient for normal testis development (Figure 1.2). However, a possible functional redundancy between *Sox9* and other members of the SoxE family, such as *Sox8* or *Sox10*, has been suggested in testis differentiation (Chaboissier et al., 2004; Barrionuevo et al., 2006).

Sox9 is initially expressed at low levels in the gonad of both sexes around 10.5 dpc, and thus before the onset of *Sry* expression (Morais da Silva et al., 1996). Furthermore, *Sox9* expression persists in Sertoli cells throughout life after transient *Sry* expression has ceased, indicating that other factors are involved besides *Sry* in regulating *Sox9* expression. Several studies have suggested that *Sf1* is particularly important for testis differentiation and might be involved in the early regulation of *Sox9* expression. Indeed, early sex-independent expression of Sox9 is abolished in the gonads of XY $Sf1^{-/-}$ mice (Sekido et al., 2004). Moreover, ubiquitous misexpression of *Sry* in XX gonads only results in the initiation of *Sox9* expression in SF1 positive cells (Kidokoro et al., 2005). Recently, the regulatory element responsible for the testis-specific expression of *Sox9* has been identified 13 kb upstream of the transcription start site, and named testis-specific enhancer of Sox9 (TES) including a 1.3 kb core region (TESCO) (Sekido and Lovell-Badge, 2008). This element is highly conserved between mouse, rat, dog and human. Chromatin immunoprecipitation (ChIP) analyses demonstrated that both SF1 and SRY bind to the TESCO enhancer sequence, confirming *Sox9* as

a direct target of SRY. Subsequent analyses with transgenic mice carrying mutations in either SRY or SF1 binding sites showed little effect on the enhancer activity but simultaneous mutation of all the binding sites completely abolished TESCO activity (Sekido and Lovell-Badge, 2008). *In vitro* co-transfection experiments demonstrated that SF1 on its own can activate TESCO weakly, whereas the combination of SF1 and SRY results in a stronger activation. These results indicate that SF1 can indeed initiate TESCO (and therefore *Sox9*) expression on its own and the synergistical effect of SF1 and SRY results in the up-regulation of its expression. Moreover, the ChIP assays revealed that SOX9 itself can bind to TESCO via the same binding sites as SRY or via fragments containing SF1 binding sites (Sekido and Lovell-Badge, 2008). This result suggests that SF1 and SOX9 might physically interact to regulate TESCO activity. Indeed, a direct interaction between the N-terminal domain of SOX9 and the C-terminal region of SF1 was previously described for the regulation of the *Amh* promoter (De Santa Barbara et al., 1998). Subsequent *in vitro* co-transfection assays showed that the TESCO element can be activated robustly by the combination of SF1 and SOX9, indicating a positive feedback mechanism in which SOX9 ensures the maintenance of its own expression. All these data led to the proposal of a new model of *Sox9* regulation via the TESCO element: (i) SRY-independent initiation, (ii) SRY-dependent up-regulation and (iii) SRY-independent maintenance (Sekido and Lovell-Badge, 2008) (Figure 1.3).

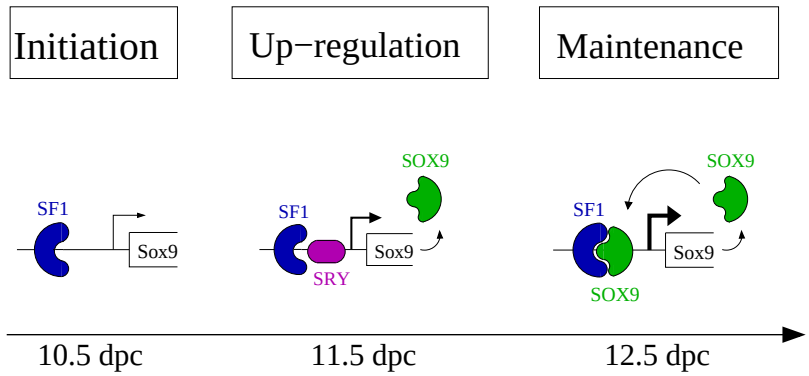


Figure 1.3: **Model for *Sox9* regulation via TESCO in XY gonads.** *Sox9* expression in the developing XY gonad is regulated via the TESCO element in three phases: (i) SF1 is binding to the enhancer at 10.5 dpc and initiates a low expression of *Sox9*, (ii) *Sry* is activated around 11.5 dpc and up-regulates *Sox9* expression (iii) SOX9 itself can bind to its own enhancer and maintains its expression via this autoregulation and other feedback loops involving FGF9 and PGD_2 signalling.

A number of other factors have been implicated to be involved in the regulation *Sox9* expression. For example, *Sox9* expression is lost in both $Gata^{ki}$ and $Fog2^{-/-}$ XY gonads (Tevosian et al., 2002), indicating that the GATA4/FOG2 complex might be required directly for *Sox9* transcription. Recent findings also show that FGF9 (fibroblast growth factor 9) plays an important role in maintaining *Sox9* expression (Figure 1.2). *Fgf9* is originally expressed in the gonad of both sexes and then becomes XY specific in Sertoli cells after *Sry* expression (Colvin et al., 2001). In XY mice carrying a homozygous null mutation of *Fgf9* ($Fgf9^{-/-}$), the expression of *Sry* is not affected and *Sox9* starts to be expressed normally at 11.5 dpc, which suggests that FGF9 is not involved in the initial up-regulation of *Sox9* expression (Kim et al., 2006b). However, SOX9 is not detectable anymore in the XY mutant gonads after 12.5 dpc which results in XY male-to-female sex reversal, indicating that FGF9 is involved in maintaining *Sox9* expression. This correlates with the finding that the misexpression of *Fgf9* alone does not result in

up-regulation of *Sox9* in XX gonads in chicken (Yoshioka et al., 2005). Similar to *Fgf9*$^{-/-}$, mice carrying a homozygous null mutation in the main receptor of FGF9 signalling, FGF receptor 2 (*Fgfr2*$^{-/-}$), show XY sex reversal due to a loss of *Sox9* expression after 11.5 dpc (Kim et al., 2007; Bagheri-Fam et al., 2008). Moreover, human patients with a deletion of the region containing *FGFR2* (10q26) display XY sex reversal (Wilkie et al., 1993). All these data suggests the importance of FGF9/FGFR2 signalling in the maintenance of *Sox9* expression. Moreover, it has been shown that conditional XY *Sox9*$^{\Delta/\Delta}$ mice have decreased levels of *Fgf9* at 11.5 dpc (Kim et al., 2006b), indicating that SOX9 is, in turn, necessary for *Fgf9* expression. Taken together, FGF9/FGFR2 signalling seems to be crucial for normal testis development by acting on *Sox9* expression via a positive feedback loop.

Another target of SOX9 is the prostaglandin D synthase gene (*Ptgds*) which is involved in the synthesis of Prostaglandin D2 (PGD2). SOX9 can directly bind and activate the *Ptgds* promoter whereas active PGD2 signalling in turn enhances import of SOX9 from the cytoplasm into the nucleus in an autocrine and paracrine manner (Wilhelm et al., 2005). Moreover, *ex vivo* analyses have shown that addition of PGD2 to XX gonads resulted in up-regulation of *Sox9* expression and partial masculinisation (Adams and McLaren, 2002; Wilhelm et al., 2005). This suggests that PDG2 signalling is sufficient to up-regulate *Sox9* expression in cells which did not express *Sry*. This Prostaglandin signalling pathway might be involved in the recruitment of XX cells to become Sertoli cells as seen in XX-XY chimeric mice (Palmer and Burgoyne, 1991b). On the other hand, mice carrying a targeted homozygous null mutation for *Ptgds* do not show any signs

of sex reversal indicating that PGD2 signalling is a back-up mechanism to ensure efficient Sertoli cell differentiation of all cells of the supporting cell lineage (Moniot et al., 2009).

In summary, SOX9 is the crucial factor which define Sertoli cell phenotype and function. This includes the direct or indirect repression of genes characteristic of the supporting cell precursors of the bi-potential gonad, as well as the repression of genes involved in ovarian development (see later). Thus, maintenance of *Sox9* expression is indispensable in the XY gonad in order to develop as a fully functional testis.

1.5 Determined to develop as female

1.5.1 Ovarian determining or anti-testis genes

Female development in mammals has often been considered as the default pathway of development (Jost, 1952). The bi-potential gonad develops into an ovary in the absence of *Sry*, suggesting the testis pathway to be the active pathway of gonadal formation. However, it has also been proposed that a gene in XX gonads (the Z gene) acts as a negative regulator of male sex determination (McElreavey et al., 1993). Although no exclusive female-determining gene has been found so far, several cases of partial XX female-to-male sex reversals have been described in the absence of *Sry*, in both mice and humans. In some cases the relevant genes have been identified, for example in LoF mutations in *Wnt4* (a member of the wingless family of genes), *Rspo1* (R-spondin1) and *Foxl2* (forkhead box

factor L2). This indicates that an active repression of the testicular pathway is necessary for normal ovarian development, rather than this depending entirely on a passive default pathway. Indeed, is seems that a "battle of the sexes" is happening in the developing gonad with different genes involved in tipping the balance either towards testis and male development or towards ovary and female development. The genes involved in this battle would activate other genes in their developmental pathway and simultaneously repress genes which are important for the opposite pathway.

The first significant molecular event happening in XX gonads is the down-regulation of *Sox9* expression in the precursors of the supporting cell lineage shortly before 11.5 dpc. Thereupon, these cells differentiate into female granulosa cells instead of male Sertoli cells. An important question is therefore which genes are directly responsible for the repression of *Sox9* at this stage and for maintaining its repression throughout life in granulosa cells. A number of genes have been proposed to act as anti-testis or ovarian-determining genes, including *Wnt4*, *Rspo1*, *β-catenin*, *Dax1* (Dosage-sensitive sex-reversal (DSS) adrenal hypoplasia congenita (AHC) critical region on the X-chromosome, gene 1), *Sox4*, *Foxl2* and *ERs* (estrogen receptors).

The Wnt family (Wingless-type MMTV integration site family) is a conserved group of secreted glycoproteins, which was named after the *Drosophila* segment polarity gene (*wingless*) and the first discovered vertebrate homolog (*integrated*). The extra-cellular WNT ligand can stimulate different intra-cellular transduction cascades: the canonical β-catenin dependent pathway and two non-canonical

pathways (planar cell polarity pathway and Wnt/Ca^{2+} pathway). In the canonical pathway, WNT ligands bind to the transmembrane receptor frizzled (Fz) and the co-receptors LDL-receptor related protein 5 and 6 (Lrp5/6) and the signal is then transduced to the cytoplasmic protein Dishevelled (Dsh). In the absence of WNT ligand β-catenin is phosphorylated by a multiprotein complex which leads to its ubiquitination and subsequent degradation via the proteosome. Upon binding of WNT, Dsh prevents β-catenin degradation by disrupting the protein complex needed for its phosphorylation. Stabilised β-catenin translocates into the nucleus and interacts with T-cell factor/Lymphocyte enhancer factors (TCF/LEF). This protein complex then binds to cis-regulatory elements of target genes and thereby activates Wnt-responsive genes (for a more detailed review see Komiya and Habas, 2008). On the other hand, it has been shown that LEF/TCF proteins can physically interact with co-repressors, such as Groucho and CtBP, which leads to the repression of their target genes (Roose et al., 1998). *Wnt4*, is particularly crucial for female development. In human patients, a duplication of the chromosomal region including *WNT4* (1p35) has been associated with XY sex reversal (Wieacker et al., 1996), whereas a point mutation within the *WNT4* coding region (E226G) has been reported as a LoF mutation which causes masculinisation in an XX patient (Biason-Lauber et al., 2004). In the mouse, *Wnt4* expression starts in the early gonad in the mesenchyme along the length of the mesonephros from 9.5 dpc onwards. At 11.0 dpc it is expressed in the mesenchyme of the indifferent gonad in both sexes and then becomes female-specific due to its down-regulation in the XY gonad around 11.5 dpc. *Wnt4* is strongly expressed in the mesenchymal cells of the developing Müllerian duct

but not the Wolffian duct (Vainio et al., 1999). XX embryos carrying a targeted null mutation of *Wnt4* (*Wnt4*$^{-/-}$) show partial masculinisation of the gonad, including some Leydig cell differentiation and the formation of a coelomic vessel. Moreover, the Wolffian duct is maintained in these embryos, whereas the Müllerian duct is absent (Vainio et al., 1999; Jeays-Ward et al., 2004). However, misexpression of *Wnt4* in XY transgenic embryos (Sf1:Wnt4) does not cause male-to-female sex reversal, although it does interfere with the normal formation of the coelomic blood vessel (Jeays-Ward et al., 2003; Jordan et al., 2003) (more detailed discussion of the *Wnt4* mutant phenotype in Chapter 6).

R-spondin 1 (*R-SPO1*) belongs to a family of secreted ligands and can interact with Fz/Lrp receptors via its two furin-like domains (Kim et al., 2006a). It might activate the same intracellular canonical pathway as WNT ligands, leading to a stabilisation of β-catenin (Kazanskaya et al., 2004). Thus, RSPO1 is thought to potentiate canonical WNT signalling although it could also act independently to achieve a similar effect. In human patients, homozygous LoF mutation of *R-SPO1* (R-SPO1$^{-/-}$) can lead to complete XX female-to-male sex reversal in the absence of *SRY* (Parma et al., 2006). *R-SPO1* does not seem to be necessary for testis differentiation because XY R-SPO1$^{-/-}$ patients develop normal and functional testes (Micali et al., 2005). In the mouse, *Rspo1* is expressed in somatic cells of the indifferent gonad in both sexes at 10.5 dpc and then becomes female-specific around 12.5 dpc (Parma et al., 2006). Mice with a targeted null mutation of *Rspo1* (*Rspo1*$^{-/-}$) show partial XX female-to-male sex reversal with formation of the male-specific coelomic vessels and the appearance of several seminiferous tubules (Chassot et al., 2008; Tomizuka et al., 2008). Moreover, adult

XX *Rspo1*$^{-/-}$ mice show production of testosterone, masculinised external genitalia and persistence of both, Wolffian and Müllerian ducts. However, expression of testis differentiation markers such as *Sox9*, *Fgf9* and *Dhh* cannot be detected in the mutant embryos at the time of sex determination, although expression of ovary differentiation marker genes, such as *Wnt4*, *Fst* and *Bmp2*, is reduced (Chassot et al., 2008; Tomizuka et al., 2008). Epistatic analysis showed that *Axin*, a known target gene of β-catenin is not expressed in XX *Rspo1*$^{-/-}$ embryos, while misexpression of a stable form of β-catenin rescues the XX *Rspo1*$^{-/-}$ phenotype (Chassot et al., 2008). These data indicates that *Rspo1* acts upstream of β-catenin. On the other hand, *Rspo1* expression is not affected in *Wnt4*$^{-/-}$, suggesting that *Rspo1* acts either upstream of, or in parallel to, *Wnt4* (Chassot et al., 2008). Taken together, these data suggest that both *Wnt4* and *Rspo1* play an important role in female sex determination by ensuring appropriate levels of stable β-catenin.

Stabilised β-catenin, as the downstream effector of WNT4 signalling and perhaps RSPO1, has been shown to rescue the phenotype of *Wnt4*$^{-/-}$ gonads in organ culture and the gonadal phenotype of *R-spo1*$^{-/-}$ mutant mice *in vivo* (Maatouk et al., 2008; Chassot et al., 2008). In XY gonads from a transgenic mouse carrying a dominant stabilised form of β-catenin (*Catnb*^{ex3}), *Sry* and early *Sox9* expression are unaffected and both the formation of the coelomic vessels and the early proliferation of the coelomic epithelium, which is specific for XY gonads, occur normally. However, *Sox9* expression rapidly decreases after 12.5 dpc resulting in a disruption of testis cord formation and up-regulation of female-specific markers (*Bmp2*, *Foxl2*, *Dax1*, *Wnt4*, *Fst*) in somatic cell lineages, indicating the transi-

tion from male to female development (Maatouk et al., 2008; Chang et al., 2008). On the contrary, Sertoli cell-specific deletion of β-catenin, by either Amh:Cre or Sf1:Cre, does not lead to any abnormalities in testis development, implying that it is not essential for Sertoli cell differentiation (Chang et al., 2008; Liu et al., 2009). However, in XX gonads, conditional deletion of β-catenin in SF1-positive somatic cells results in the appearance of testis-specific vasculature, maintenance of the Wolffian duct and loss of germ cells, closely resembling the phenotype of both *Wnt4*$^{-/-}$ and *R-SPO1*$^{-/-}$ mutant mice (Liu et al., 2009). Taken together, the data suggest that the role of β-catenin is to antagonise testis development and to maintain rather than initiate female development. It has been shown in chondrocyte differentiation that SOX9 and β-catenin are able to repress each others effects on transcriptional activation in a DNA binding independent manner. As the C-terminal transactivation domain of *Sox9* physically can interact with the *Armadillo* repeats in β-catenin, SOX9 can compete with LEF/TCF factors for binding to β-catenin. The resulting SOX9/β-catenin complex is eventually degraded via the proteosomal machinery (Akiyama et al., 2004). Therefore, *Sox9* can act as a de-stabiliser of β-catenin causing a reduction in the expression of β-catenin target genes or vice-versa. Similarly, there is some evidence that human SRY is able to inhibit β-catenin by direct protein interaction *in vitro* (Bernard et al., 2008). It is not known which part of the SRY protein mediates the interaction with β-catenin, only that the SRY HMG-box on its own is not sufficient. Other studies indicate a role of β-catenin in estrogen production in granulosa cells where β-catenin is able to directly interact with SF1 and thereby modulate SF1 activity, e.g. in the regulation of *Cyp19A1 in vitro* (Parakh et al., 2006).

Dax1 (also known as *Nr0b1*) is an X-linked gene encoding an unconventional member of the nuclear receptor (NR) superfamily. It lacks the classic zinc finger DNA binding domain of NRs and instead contains three and a half repeats of a novel domain composed of the LXXLL-motif (Ito et al., 1997), which appears to be involved in interactions between DAX1 and other NRs. DAX1 binds to SF1 through this motif and represses SF1 activity by the recruitment of a nuclear co-repressor NcoR (Ito et al., 1997; Crawford et al., 1998). On the other hand, the *Dax1* promoter contains binding sites for SF1 and mutation of these sites abolishes *Dax1* expression. Moreover, *Dax1* expression is significantly reduced in $Sf1^{-/-}$ mice (Hoyle et al., 2002) implying that *Dax1* is regulated by SF1 in the developing gonad. Thus, the two genes interact in a complex fashion which is not yet fully understood. A consensus DNA binding sequence for DAX1 has not yet been described. It might bind DNA directly, like other nuclear receptors (e.g. SF1), but it has also been suggested that DAX1 binds to DNA secondary structures (Zazopoulos et al., 1997) or that it does not bind to DNA, but to RNA (Lalli et al., 2000). Human XY patients with duplications of a region of Xp21, containing the *DAX1* gene, display a male-to-female sex reversal (Bardoni et al., 1994). This suggested that *DAX1* can act as an anti-testis gene and that it might be is important in ovary development. In mice, *Dax1* is expressed in the indifferent genital ridge of both sexes and is down-regulated in XY gonads during testis development (by 12.5 dpc) whereas the expression persists in the somatic cells of XX gonads throughout ovarian development (Swain et al., 1996). Misexpression of *Dax1* in mouse strains carrying a weak *Sry* allele (Y^{POS}) or with a delayed onset of *Sry* expression causes XY sex reversal (Swain et al., 1998).

Moreover, targeted deletion of *Dax1* in mice did not result in XX sex reversal (Meeks et al., 2003). On the other hand, mice carrying this *Dax1* deletion show XY male-to-female sex reversal in the presence of Y^{POS} (Meeks et al., 2003) or on a specific genetic background (C57BL/6HJ) (Bouma et al., 2005), indicating that *Dax1* is required for testis development. This is in contrast to the human cases where LoF mutations in *DAX1* do not affect testis development in XY AHC patients (Zanaria et al., 1994; Muscatelli et al., 1994). The molecular mechanisms underlying this paradox are not understood yet, but it is possible that *Dax1* is needed for both testis and ovarian development depending on timing and dosage of its expression. It is possible that the deletion might not be a true null mutation as only the second of the two *Dax1* exons was deleted and a truncated DAX1 protein (containing only the N-terminal region) could still retain some functional activity (Ludbrook and Harley, 2004). It might interact with SF1 via its remaining N-terminus and could act as a dominant negative with respect to SF1 activity (maybe by reducing the pool of SF1 protein available to activate the male pathway).

Sox4 belongs to the SOX family of transcription factors (Gubbay et al., 1990), subgroup C, together with *Sox11*. *Sox4* is expressed in a number of tissues including neural tissue, heart, lung and thymus (van de Wetering et al., 1993). Moreover, in mice *Sox4* has been found to be expressed specifically in the ovary and not in the testis at the time of sex determination (P. Koopman, personal communication) suggesting a potential role in ovarian development. Mice with a homozygous null mutation of *Sox4* ($Sox4^{-/-}$) die at 14.0 dpc due to heart malformation (Schilham et al., 1996) but the gonadal phenotype of those mutant mice

has not yet been analysed. It is known that SOX proteins are transcriptional regulators that can activate or repress target genes according to the cellular context (Chew and Gallo, 2009). Hence, it is possible that SOX4 could act as a repressor of *Sox9* expression in the developing XX gonad. Moreover, it has been shown that SOX4 directly interacts with β-catenin and TCF/LEF proteins and that SOX4 can enhance β-catenin activity in colon carcinoma cells *in vitro* (Sinner et al., 2007).

Foxl2 encodes for a member of the forkhead family of transcription factors. It was originally identified as a candidate for a gene involved in ovarian development in goats. In these animals, an 11.7 kb deletion 200 kb upstream of the *Foxl2* gene causes a dominant hornless phenotype that is associated with a recessive XX female-to-male sex reversal, known as polled/intersex syndrome (PIS) (Vaiman et al., 1996). In human patients, LoF mutations of *FOXL2* cause the dominant disorder blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), characterised by small palpebral fissures (blepharophimosis), drooping eyelids (ptosis), a small skinfold inwards from the lower lid (epicanthus inversus) and a broad nasal bridge (Crisponi et al., 2001). Two distinct types of BPES have been described: BPES type I which is associated with premature ovarian failure (POF) in female patients and BPES type II where patients remain fully fertile. Mutations within the coding sequence of *FOXL2* resulting in a truncated FOXL2 protein cause BPES type I, while frameshifts or duplications further downstream in the sequence produce an extended protein which is associated with BPES type II. Some BPES patients show intact coding sequences for *Foxl2*, but carry mutations about 100 kb upstream of the gene itself (Uhlenhaut and Treier, 2006). In the

mouse, *Foxl2* is, amongst other tissues, expressed in the granulosa cells of ovarian follicles. The gonadal expression is female-specific and starts in the supporting cell precursors of the ovary around 12.5 dpc and is maintained in adult granulosa cells (Schmidt et al., 2004). *Foxl2* is also expressed in some theca cells in the adult ovary (Uhlenhaut et al., 2009). Mice with a heterozygous mutation of *Foxl2* display no phenotype, whereas homozygous loss of *Foxl2* results in a number of defects resembling the human BPES condition (Uda et al., 2004; Schmidt et al., 2004). This includes XX infertility and the postnatal up-regulation of testis-specific genes (more detailed description of the *Foxl2* mutant phenotype in Chapter 5). Only a few target genes of FOXL2 have been described so far, including the gonadotrophin releasing hormone receptor (GnRHR), the human steroidogenic acute regulatory gene (*StAR*), which is involved in the rate-limiting step of steroidogenesis, and *Cyp19* which encodes for an aromatase involved in converting androgens into estrogens (more detailed discussion of FOXL2 target genes in Chapter 4). Moreover, it has been reported that forkhead factors can compete with TCFs for interaction with β-catenin *in vitro* and direct interaction of forkhead factors and β-catenin leads to the enhancement of forkhead transcriptional activity (Hoogeboom et al., 2008).

Estrogen receptors (ERs) are members of the nuclear receptor superfamily which transmit 17β-estradiol (E_2) signals into the nucleus and activate target genes by binding to estrogen response elements (ERE). EREs are palindromic sequences of the estrogen receptor half site GGTCA (Klinge, 2001). Two ERs have been identified in mammals which are expressed in many tissues including the ovaries, where *ER*α is expressed in stroma, theca and granulosa cells and *ER*β is expressed

predominantly in granulosa cells (Fitzpatrick et al., 1999). Single mutations of either *ERα* or *ERβ* cause distinct defects. In XX $ER\beta^{-/-}$ mice, follicular development is partially arrested and follicular maturation occurs at reduced efficiency resulting in smaller ovaries and sub-fertility whereas XY males are fully fertile (Krege et al., 1998). XX $ER\alpha^{-/-}$ mice are infertile with a block of folliculogenesis and a decrease in granulosa cell proliferation, severe uterine and vaginal hypoplasia and unovulatory adult ovaries with accumulation of large cystic follicles. Moreover XY $ER\alpha^{-/-}$ mice are infertile indicating that estrogens might play an important role in male as well as in female development (Lubahn et al., 1993; Dupont et al., 2000). Mice carrying double homozygous mutations of both estrogen receptors ($ER\alpha\beta^{-/-}$) display a more severe phenotype than the single mutants (Couse et al., 1999; Dupont et al., 2000). Similar to $ER\alpha^{-/-}$ males, XY $ER\alpha\beta^{-/-}$ mice are infertile due to reduced numbers of sperm, while the reproductive tract appears normal. XX $ER\alpha\beta^{-/-}$ mice are also infertile and show vaginal hypoplasia, large ovarian cysts, absence of mature corpora lutea and proliferation defects in granulosa and theca cell layers. These mice also show partial sex reversal with appearance of seminiferous tubule-like structures and Sertoli-like cells expressing *Sox9* and *Amh*. However, this phenotype only occurs after puberty (Couse et al., 1999). It has also been shown that forkhead transcriptional regulators are essential for estrogen receptor DNA binding, e.g. in breast cancer cell lines (Carroll et al., 2005), suggesting a possible link between FOXL2 and ERs.

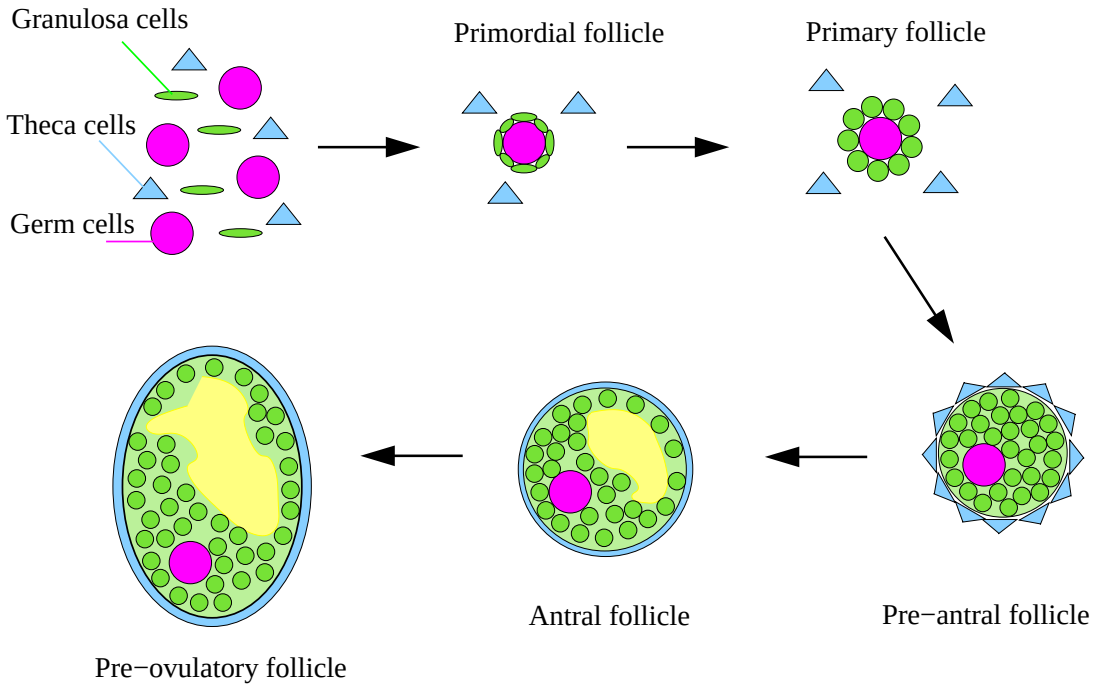
1.5.2 Ovarian development

The germ cells in the XX genital ridge enter meiotic prophase at 13.5 dpc and arrest in diplotene at the time of birth. It has been proposed that they follow an intrinsic clock to enter meiosis, as even germ cells assembled in lung aggregates enter meiosis about 13.5 dpc *in vitro* (McLaren and Southee, 1997). However, it has also been shown that not all germ cells in the ovary enter meiosis at the same time but in an anterior-to-posterior pattern (Menke et al., 2003; Bullejos and Koopman, 2004). Moreover, there is now substantial evidence that suggests that retinoic acid, synthesised by the mesonephros, diffuses into the early ovary to trigger entry of the germ cells into meiosis, probably by induction of *Stra8* expression (Bowles et al., 2006; Koubova et al., 2006). In the early testis, CYP26B1, which is produced by Sertoli cells and probably a target of *Sox9*, degrades retinoic acid and thus prevents meiosis (Bowles et al., 2006; Koubova et al., 2006).

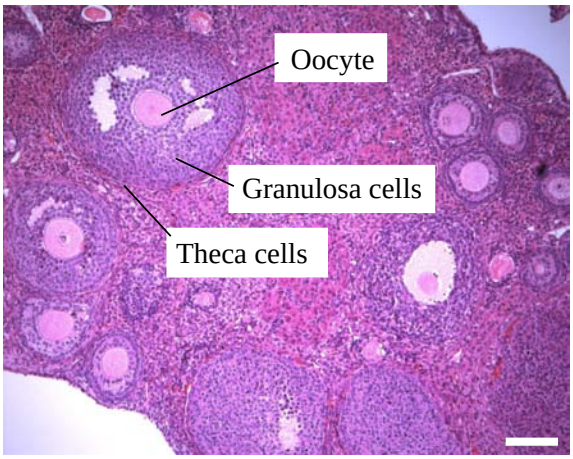
Morphological changes in XX gonads are not obvious until late gestation when the ovary can be divided into two main regions: the cortex and the medulla. Around 16.5 dpc many germ cells in the medulla undergo apoptosis resulting in an accumulation of germ cells in the cortex (Yao et al., 2004). The germ cell loss correlates with the breakdown of germ cell cysts and the start of folliculogenesis (Pepling and Spradling, 2001). At this stage, the pre-granulosa cells extend cytoplasmic processes and enclose individual oocytes in a single layer of squamous cells, forming primordial follicles (Figure 1.4). Oocytes express *Figα* (factor in germ line α), which is involved in the recruitment of pre-granulosa cells. Although germ cells of mice with a homozygous null mutation in *Figα* enter meiosis cor-

rectly, they fail to form primordial follicles resulting in the depletion of oocytes directly after birth (Soyal et al., 2000). During normal ovarian development, the primordial follicles enlarge after birth due to an increase in the size of the oocyte and the transition of the squamous granulosa cells into cuboidal granulosa cells, resulting in the formation of primary follicles (Hirshfield, 1991). The granulosa cells then proliferate to form multiple layers around the oocyte and thereby form pre-antral follicles, which are enclosed by an outer layer of theca cells. During the following antral stage, the granulosa cells secrete follicular fluid (containing serum proteins and steroid hormones) which fills the emerging antral cavity. Most of the antral follicles will undergo atresia, but the ones remaining will become pre-ovulatory follicles under the influence of follicle-stimulating hormone (FSH). After ovulation, the remaining granulosa and theca cells form the corpus luteum which secretes steroid hormones to promote implantation of the embryo. In the adult ovary, follicles of each stage can be found, which is due to some primordial follicles entering a temporary state of quiescence to ensure the continuous production of ovulatory follicles (Figure 1.4).

The function of the female steroidogenic cell lineage, the theca cells, is the production of estrogens. Theca cells express *Cyp11a*, *3β-HSD* (*HSD3B2*) and *Cyp17* which are required for the conversion of cholesterol into androgens. Granulosa cells express *Cyp19* and *17β-HSD* (*HSD17B1*) which converts these androgens into estradiol. Thus, both cell types are required for estrogen production in the ovary (Magoffin, 2005).



(a)



(b)

Figure 1.4: **Model of ovarian follicle development.**
(a) Folliculogenesis starts in the embryo with a pool of germ cells and somatic cells (granulosa and theca cells). The model shows the development of primordial follicles which grow to primary, pre-antral and antral stages before they become pre-ovulatory follicles. The granulosa cells (green) surround the oocyte (pink) and proliferate to several layers of supporting cells. The theca cells (blue) start to surround the follicles at the pre-antral stage. The antral cavity which contains the follicular fluid is shown in yellow. Adapted from Barnett et al. (2006). (b) H+E staining illustrating the morphology of an adult ovary. Scale bar = 100μm.

1.6 Outline of this thesis

Sox9 plays an essential role during embryonic development as its presence or absence determines the fate of the gonadal supporting cell lineage and thereby controls the establishment of either a testis or an ovary. The aim of this thesis is to investigate the effect of potential ovarian-promoting or anti-testis genes on the expression of *Sox9* via the TESCO element, both *in vitro* and *in vivo*. Therefore, the repressing potential of DAX1, SOX4, FOXL2, ERα and WNT4-signalling on TESCO activity was individually analysed in transfection assays *in vitro*. Subsequently, the consequences of loss of either *Foxl2* or *Wnt4* individually or in combination was assessed *in vivo*, focusing on the potential de-repression of TESCO and the male pathway in XX gonads during embryonic development and adult stages. Finally, different possible mechanisms by which FOXL2 could mediate repression of TESCO were examined *in vitro*.

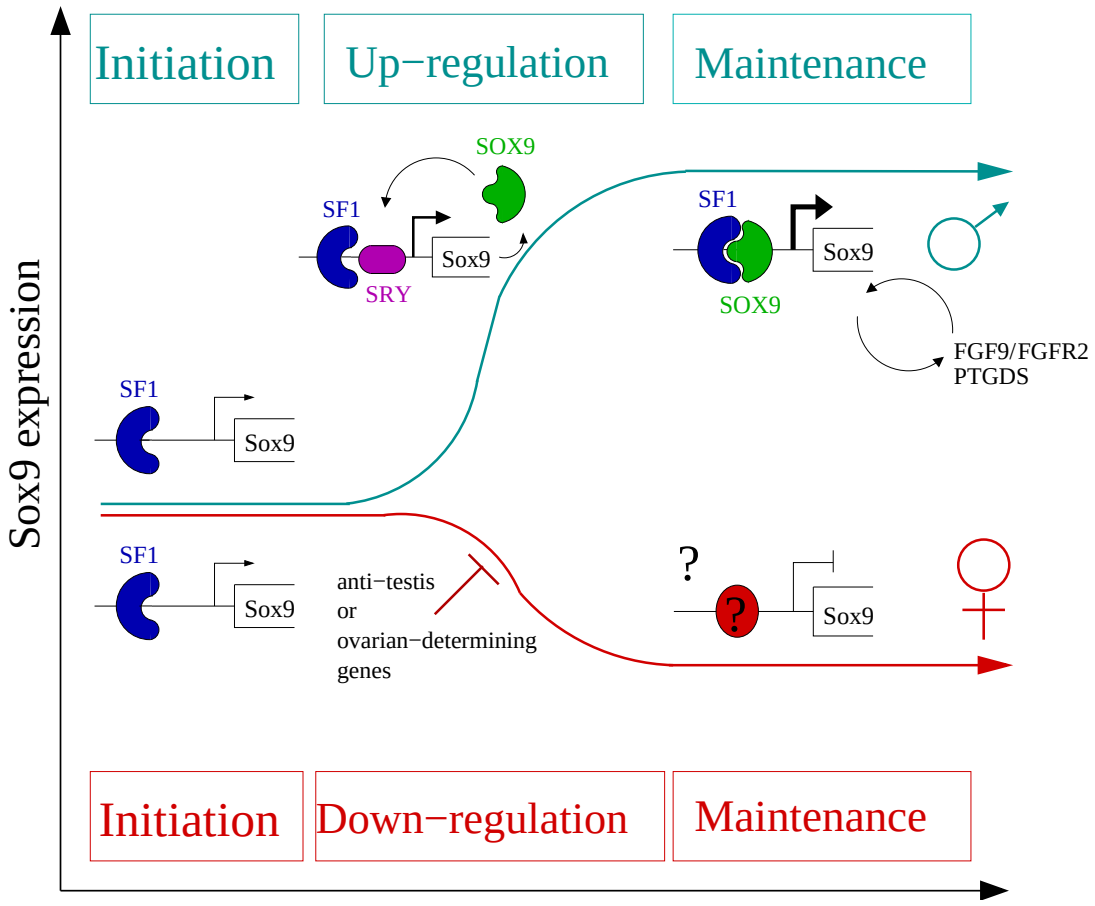


Figure 1.5: **Model of distinct *Sox9* regulation in XX and XY gonads during early development.**
In the bi-potential gonad, SF1 binds to the TESCO enhancer of *Sox9* and initiates a low *Sox9* expression in both sexes. In XY gonads, SRY binds to TESCO and up-regulated *Sox9* expression, which is maintained by SOX9 itself and other factors such as FGF9 and PGD_2 signalling. In contrast, the initial *Sox9* expression is actively down-regulated in XX gonads and the repression is maintained throughout life. Several anti-testis or ovarian-determining genes have been proposed to be involved in this regulation of *Sox9* expression, although no direct interactions have been shown yet.

Chapter 2

Materials and Methods

2.1 Sequence analysis

TESCO sequences from different species were compiled from the Ensemble database (`http://www.ensembl.org`): mouse sequence (ENSMUSG00000000567), rat sequence (ENSRNOG00000002607), human sequence (ENSG00000125398) and dog sequence (ENSCAFG00000004374). The mouse TESCO sequence was analysed for potential transcription factor binding sites using the MatInspector program (`http://www.genomatix.de`). To analyse the homology between the TESCO elements of different species, sequences were aligned and compared using the BLAST software from NCBI (`http://www.blast.ncbi.nlm.nih.gov/Blast.cgi`).

2.2 Luciferase Assay

2.2.1 Cloning of constructs

To analyse the effect of several candidate genes in co-transfection assays, the cDNAs of those genes were individually cloned into a CMV expression vector (for plasmid maps see Appendix). Existing plasmids containing the cDNA of interest were cut by enzymatic digestion following standard protocols. For *Foxl2*, the gene was amplified from whole ovarian DNA by PCR (KOD Hot Start Polymerase, Novagen) following standard protocols. The DNA fragments were then purified using the Geneclean II kit (Qbiogene) and ligated into the linearised pcDNA3 vector (Invitrogen) for 30 minutes at 16°C. Subsequently, constructs were transformed into chemically competent TOP-10 cells (Invitrogen) by adding the ligation mix to 50μl of TOP-10 cells, incubating on ice for 20 minutes followed by an incubation at 42°C for 1 minute. After resting for 5 minutes on ice, 500μl of LB medium was added to the cells, which were then incubated at 37°C for 1 hour. 100μl of transformed cells were streaked onto LB plates containing ampicillin (50μg/ml) and incubated at 37°C overnight. The next day, single bacterial colonies were picked and incubated in 2ml LB broth containing ampicillin (100μg/ml) at 37°C in a Brunswick Scientific Shaking Incubator for 6 hours at 350rpm. Plasmid DNAs were extracted via Miniprep following standard protocols and successful cloning was determined by restriction digest. For large-scale DNA preparations, 100ml LB broth containing ampicillin (100μg/ml) were inoculated with 100μl of the remaining Mini-culture from the correct clones and incubated overnight at 37°C. The plasmid DNAs were extracted via Maxipreps

using the Nucleobond Xtra kit (Macherey-Nagel) and stored at 4°C. From each clone, 1ml of culture was kept and 0.5ml 50% glycerine was added to generate glycerine stocks which were stored at -80°C.

For analyses of transcription factor binding, several base pairs were mutated in the TESCO element. This was done using modified oligonucleotides in a PCR amplification of the TESCO sequence using the KOD polymerase, resulting in specific point mutations at the sites of interest. The TESCO sequence with all FOX/ERE sites mutated was generated by *de novo* DNA synthesis (GENEART, Germany). The altered TESCO sequences were then cloned into the Luciferase vector (pδ51LUCII) between BamHI and SalI. All constructs were sequenced (Cogenics) and analysed to determine the accuracy of the mutations and cloning.

2.2.2 Cell culture

COS7 cells (ATTC #CRL-1657) were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) with 10% fetal calf serum (FCS, Gibco), 1% L-glutamine (Gibco) and 1% Penicilin/Streptomycin (Gibco) at 37°C and 5% CO_2. When the cells were 70-80% confluent, they were washed with PBS (calcium and magnesium free, Gibco) and incubated with Trypsin-EDTA (Gibco) until they detached from the bottom of the flasks. Culture medium was added to stop trypsinisation and the cells were centrifuged at 1000rpm for 5 minutes at 4°C. The cell pellet was then resuspended in fresh culture medium and the cells re-plated at a lower density. For stock freezing, cells were re-suspended in 0.5ml of DMEM containing 20% FCS and 10% DMSO (Sigma) and stored in liquid nitrogen.

2.2.3 Transfection assays

COS7 cells were plated in normal culture medium at 5x10^4 cells per well in 24-well plates 24 hours before transfection. The cells were then transfected with 0.5μg Luciferase vector containing the TESCO element (either wild-type or with inserted point mutations) and 0.1μg of the expression vectors containing *Sf1*, *Sox9* or *Sry* to activate Luciferase activity. To analyse potential repression effects, the same amount of expression vectors containing *Foxl2*, *ER*α, *Dax1*, *Sox4*, β-*catenin*, *Tcf1*, *Tcf3*, *Tcf4* or *Lef1* were added individually in different co-transfection assays. To determine dosage-dependent effects, increasing amounts of these effector genes were transfected (20ng, 60ng, 180ng, 540ng). The amount of expression vector was equalised by adding pcDNA3 vector, the amount of DNA was adjusted using pBluescript vector. For normalisation 0.3μg pmiwZ (Suemori et al., 1990) was added. The DNA and 4μg Lipofectamine (Invitrogen) were mixed in serum-free OptiMEM (Gibco), incubated at room temperature for 20 minutes and then added to the cells in fresh DMEM. Luciferase activity was measured 48 hours after transfection using the Luciferase assay system (Promega) and normalised to β-galactosidase activity using 2.5μg of o-Nitrophenyl-β-D-galactopyranosid substrate (ONPG, Sigma) per ml of LacZ buffer (10mM KCl, 1mM $MgCl_2$, 40mM NaH_2PO_4, 60mM Na_2HPO_4, 1mM DTT). All co-transfection assays were performed in triplicates, error bars indicate the standard deviation from three biological replicates and p-values were calculated using the GraphPad Software t-test calculator (www.graphpad.com/quickcalcs/ttest1.cfm).

2.3 Mouse Lines

2.3.1 Husbandry

Animals were kept on a 12 hour light-dark cycle, water and food were provided *ad libitum*. For embryonic studies, the morning of vaginal plug (VP) detection was defined as 0.5 days *post coitum* (dpc), assuming the time of conception was in the middle of the dark phase. For postnatal studies, the day when pups were born was defined as postnatal day zero (P0), followed by the first day *post partum* (dpp). All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 under the project licence 80/1949A and the personal licence 80/10095.

2.3.2 Genotyping

Ear pieces were lysed overnight at 55°C in lysis buffer (0.5M Tris-HCl pH 8.5, 5mM EDTA, 0.1M NaCl, 2,5% SDS) with 1mg/ml proteinase K (Roche). The DNA was then purified by Phenol extraction using an equal amount of Phenol-Chloroform-Isoamylalcohol (25:24:1) followed by precipitation with NaOAc (1/10 volume) and Isopropanol (1x volume). Finally, the DNA was resuspended in 50μl TE and used in PCRs. For genotyping of embryos or newborn pups, a small piece of tail was used and the DNA extracted as described above.

2.3.3 Mouse lines

TESCO:CFP

The 1.3 kb TESCO sequence was cloned into the Asshsp68LacZpA vector, containing the hsp68 minimal promoter and in which the *LacZ* gene had been replaced by ECFP. The TESCO element drives CFP expression specifically in Sertoli cells of the testis (Sekido and Lovell-Badge, 2008). The mice were kept on an MF1 background and the presence of the transgene was detected by standard PCR with primers located in the ECFP: 5'-GACCCTGAAGTTCATCTGCAC-3' and 5'-GTGGCTGATGTAGTTGTACTC-3' (for protocol see Appendix 8.2).

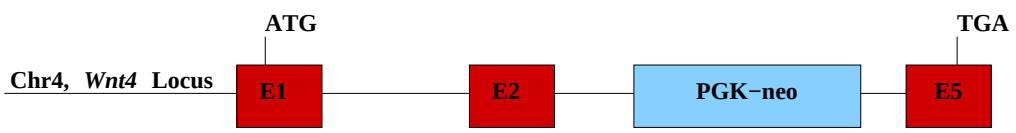
Foxl2^{LacZ}

Foxl2^{LacZ} mice were originally generated and described by Schmidt et al. (2004). *Foxl2* is a single exon gene and encodes for a protein of 375 amino acids. Upon homologous recombination at the *Foxl2* locus, the amino acids 62 to 375 were replaced by the *LacZ* gene, generating a functional null allele (see diagram below). The mice were first kept on a C57BL/6 background, but later bred onto an MF1 background. Homozygous offspring were obtained by inter-crossing heterozygous males and females. The Foxl2^{LacZ} mice were genotyped by detecting the wild-type *Foxl2* allele with the primer set (5'-CAGATGATGGCCAGCTACCCCGAGC-3' and 5'-GTTGTGGCGGATGCTATTCTGCCAGCC-3') and the mutant allele

with a third primer (5'-GTAGATGGGCGCATCGTAACCGTGC-3') which is located within the LacZ sequence and the forward primer described above (for protocol see Appendix 8.2).

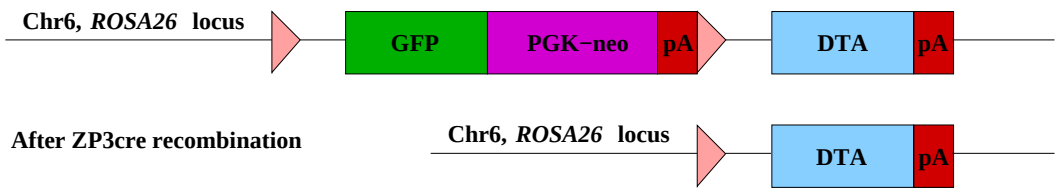
Wnt4^{neo}

Wnt4^{neo} mice were originally generated and described by Stark et al. (1994). Homologous recombination at the *Wnt4* locus resulted in a deletion of about 2.2 kb including the coding sequence for the WNT4 amino acids 106 to 196 and replacement by a PGK-neo selection cassette, resulting in a functional null allele (see diagram below). The mice were kept on an 129/Sv background and homozygous offspring were generated by inter-crossing heterozygous mice. The wild-type *Wnt4* allele was detected with the primers 5'-CTTCACAACAACGAGGCTGGCAGG-3' and 5'-CACCCGCATGTGTGTCAAGATGG-3', whereas the targeted *Wnt4*^{neo} allele was identified by a primer located within the sequence of the PGK-neo cassette 5'-GCATTGTCTGAGTAGGTGTCATTC-3' and the reverse primer described above (for protocol see Appendix 8.2).



R26DTA

R26DTA mice were originally generated by Ivanova et al. (2005) and contain a GFP/PGK-neo cassette between two LoxP sites followed by the diphtheria toxin fragment A (DTA) in the ROSA26 locus. In the absence of Cre recombinase, GFP is expressed and DTA is not active. Upon Cre recombination, the GFP/PGK-neo cassette located between the two LoxP sites (pink triangles in the diagram below) is excised resulting in the activation of DTA. When expressed, the subunit A of the diphtheria toxin mediates the termination of all protein synthesis which eventually leads to apoptosis of the cell (Maxwell et al., 1986), therefore resulting in cell depletion specifically in the cell type expressing the Cre. Homozygous R26DTA mice were kept on the C57BL/6 background and bred to mice carrying a Cre recombinase under the control of the oocyte-specific promoter of the zona pellucida 3 gene (Zp3:Cre, Fvb background) to generate Zp3:Cre, R26DTA mice. The presence of the Zp3:Cre allele was detected by PCR using the primer set 5'-GGCGGATCCGAAAAGAAAA-3' and 5'-CAGGGCGCGAGTTAGTAGC-3' (for protocol see Appendix 8.2).



2.3.4 Harvesting of gonads

For the analysis of embryonic gonads, pregnant mice were harvested using a schedule 1 method at different time points after VP. Embryonic gonads (including the mesonephros at stages earlier than 16.5 dpc) were then dissected into PBS and cleaned from all other tissues. In addition, a small piece of embryo tail was collected and used for genotyping. For analyses of postnatal stages, pups and adult mice were harvested at different ages using a schedule 1 methods and ovaries and testes were then dissected into PBS as described above.

2.4 Immunohistochemistry

2.4.1 Preparation of samples

Embryonic gonads were fixed in 4% PFA (w/v in PBS) for 30 minutes (from 12.5 dpc to 14.5 dpc) or 1 hour (from 16.5 dpc to P0) at 4°C. Postnatal gonads were fixed overnight at 4°C. After fixation, gonads were washed with PBS and equilibrated in 30% sucrose (w/v) at 4°C overnight. Samples were then embedded in OCT compound (VWR) in Dispomoulds and frozen using dry ice. Frozen samples were sectioned at a thickness of 8μm using a Leica cryostat (model CM3050S) and the sections were then collected onto Superfrost Plus slides (Thermo Scientific). Slides were stored at -80°C until further processing.

2.4.2 Histology

For Haematoxylin-Eosin (H+E) staining, postnatal gonads were fixed in Bouin's solution (Sigma) overnight at 4°C. After fixation, samples were washed in 70% ethanol and then stored in fresh 70% ethanol at 4°C. Samples were subsequently embedded in wax, sectioned at a thickness of 5μm and processed for H+E staining (Histology Service, NIMR).

2.4.3 Immunostaining

Cryosections were thawed at room temperature and then washed twice with PBS to remove the remaining OCT compound. Adult samples were additionally fixed in 4% PFA for 10 minutes at room temperature and then washed again twice with PBS. Afterwards, sections were blocked with 1% normal donkey serum (Sigma) in PBS/0.05% triton for 1 hour at room temperature. Subsequently, sections were incubated with primary antibodies diluted in blocking solution in a humidified chamber overnight at 4°C. The following day, sections were washed three times with PBS/0.1% triton and then incubated for 1 hour with the appropriate fluorescent secondary antibody (Molecular probes) diluted in blocking solution in a humidified chamber in the dark at room temperature. After the slides were washed with PBS/0.1% triton three times, sections were incubated with DAPI solution (Sigma, 1:10000 v/v in PBS) at room temperature for 5 minutes. Finally, sections were rinsed with PBS, air dried and mounted with Aqua PolyMount solution (Polysciences).

For co-staining, sections were washed three times in PBS/0.1% triton after incu-

bation with the secondary antibody and then fixed again in 4% PFA for 10 minutes at room temperature. After rinsing with PBS, sections were blocked for 1 hour at room temperature and the second primary antibody was added in blocking solution and incubated overnight at 4°C. The following day, sections were processed as described for single staining.

Primary antibodies

Protein	Host	Dilution	Source
GFP	Rabbit	1:500	Invitrogen
GFP	Goat	1:500	Abcam
SOX9	Goat	1:250	R&D systems
SOXE	Rabbit	1:1000	Morais da Silva et al. (1996)
FOXL2	Goat	1:500	Imgenex
β-galactosidase	Mouse	1:500	Promega

Secondary antibodies

Antibody	Fluorescence	Dilution	Source
Goat anti-rabbit	Alexa-488	1:500	Molecular Probes
Donkey anti-goat	Alexa-488	1:500	Molecular Probes
Donkey anti-goat	Alexa-555	1:500	Molecular Probes
Goat anti-mouse IgG2	Alexa-594	1:500	Molecular Probes

2.4.4 Imaging

Pictures of H+E stainings were obtained with a light microscope (Leica CTR-MIC) using a 40x lens and OpenLab software (Improvision, UK). Immunofluorescent pictures were taken with a Leica SP5 confocal using a LAS software (Leica, Germany). Images were taken with either 40x or 100x lenses and processed using Adobe Photoshop 5.5 (Adobe Systems).

2.5 Protein interaction assays

2.5.1 *In vitro* translation of ^{35}S-SF1

^{35}S-labelled SF1 was generated following the instructions of the TNT coupled reticulocyte lysate system from Promega. The pcDNA3-Sf1 plasmid was used as DNA template (0.5μg/μl). For the reaction mix, 25μl TNT rabbit reticulocyte lysate, 2μl TNT reaction buffer, 1μl RNA Polymerase T7, 1μl 1mM amino acid mixture (without methionine), 1μl RNase Inhibitor, 2μl DNA template and 1μl [^{35}S]methionine (10.25mCi/ml) were incubated in a total volume of 50μl at 30°C for 90 minutes. The reaction was then stopped by transfer to ice and a fraction of the *in vitro* translated protein was subsequently analysed on a 6% acrylamide gel whereas the remaining sample was aliquoted and stored at -80°C. The gel was run at 170V for 2-3 hours, dried afterwards and the presence of radioactivity was detected by film (Kodak) exposure overnight at -80°C.

2.5.2 Preparation of GST-FOXL2

The *Foxl2* cDNA was cloned into the GST gene fusion vector pGEX-2T (Addgene) via BamHI and EcoRI. The construct was transformed into bacterial cells and the following day clones were picked and inoculated in 5ml LB broth at 37°C overnight. Cultures were then transferred to 150ml fresh LB broth and further incubated at 30°C until it reached an OD between 0.5 - 1.0 after approximately 4 hours. Protein production was induced by adding 0.2mM IPTG followed by incubation at 30°C for 2 hours. Subsequently, cultures were centrifuged at 5000rpm for 10 minutes at 4°C. Pellets were resuspended in 5ml PBS (including 100μM $ZnCl_2$, 1mM PMSF) and sonicated (duty cycle 30%, output control 5, 30 seconds on, 1 minute off, 7 cycles). Afterwards Tween-20 and DTT were added to a final concentration of 1% and 4mM, respectively. Samples were then centrifuged at 4000rpm for 15 minutes at 4°C. The supernatant was incubated with 0.25ml Glutathione Sepharose-4B beads (Pharmacia, 50% w/v in PBS) by mixing gently for 10 minutes at 4°C. The beads were then washed three times with PBS (containing 100μM $ZnCl_2$, 1mM PMSF) and centrifuged at 800rpm for 2 minutes at 4°C. Afterwards, the beads were resuspended in buffer G (50mM Tris-HCl pH9.5, 5mM Glutathione S-transferase (Sigma)) and mixed gently for 15 minutes at 4°C. Finally, the beads were collected by a short spin up to 5000rpm at room temperature and the supernatant was transferred into a dialysis tube. The protein extracts were dialysed against 10mM Tris-HCl pH 8.0, 100mM $ZNSO_4$, 1mM PMSF overnight at 4°C. The final protein extracts were aliquoted and stored at -80°C.

2.5.3 Co-Immunoprecipitation

5μl ^{35}S-SF1 and either 3μg GST-FOXL2 or GST control protein were incubated in CoIP buffer (20mM HEPES pH7.9, 100mM KCl, 10% Glycerol, 0.1% Triton, 1mM DTT, 1mM PMSF) at 4°C for 2 hours. Samples were subsequently incubated with 20μl Glutathione Sepharose-4B beads (50% w/v in PBS) for 30 minutes at 4°C. The beads were then washed three times with CoIP buffer and centrifuged at 800rpm for 2 minutes at 4°C. Afterwards, the beads were resuspended in 20μl 2x protein sample buffer (Invitrogen) and boiled at 95°C for 5 minutes. 10μl of each sample and 5μl input control (^{35}S-SF1 protein) were run on 4-12% pre-made gels (Invitrogen) for 1 hour at 170V and 70mA. Afterwards, the gels were dried for 30 minutes and the presence of radioactive sample was detected by film exposure overnight at -80°C.

2.5.4 Nuclear protein extract

COS7 cells were plated at a density of 5x10^4 cells per 10 cm^2 dish and incubated at 37°C and 5% CO_2. The following day, 24μg of either pcDNA-Foxl2 or pcDNA-Sf1 were transfected using 60 μg Lipofectamine in fresh medium. After 48 hours, the cells were washed twice with PBS and then centrifuged for 15 seconds at 13000rpm at 4°C. The cells were resuspended in 600μl ice cold buffer A (10mM HEPES pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1x proteinase inhibitor cocktail) and incubated on ice. After 15 minutes, 37.5μl 10% NP-40 were added to the cells, which were vortexed for 10 seconds and then centrifuged for 30 seconds at 13000rpm at 4°C. The supernatant, which contains

the cytoplasmic proteins, was removed and the pellet resuspended in 50μl ice cold buffer C (20mM HEPES pH7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1x proteinase inhibitor cocktail). After incubation at 4°C for 15 minutes, the samples were centrifuged for 5 minutes at 13000rpm at 4°C. The supernatant containing the nuclear cell extract was then aliquoted and stored at -80°C.

2.5.5 Electrophoretic Mobility Shift Assay (EMSA)

Two complementary DNA sequences of a length of 43 nucleotides, each containing a 5' overhang including a GTP, were annealed to form a double strand. 600pmol of each oligonucleotide (MWG) were incubated in 100μl STE (100mM NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA) for 10 minutes at 95°C, followed by 30 minutes at 65°C, 1 hour at 37°C and overnight at 4°C. The double stranded sequences were then labelled by filling the 3' gaps of each strand with nucleotides including [α-^{32}P]dCTP by Klenow polymerase in a total volume of 50μl for 30 min at 37°C (50ng DNA, 5μl 10xbuffer, 1μl 5mM d(GAT)P, 2μl d^{32}P-αCTP (10mCi/ml), 1μl Klenow DNA polymerase I (5U/μl)). The reaction was stopped by phenol-chloroform extraction and 50μl STE were added before the labelled probes were purified over a Sephadex G50 column. The radioactive incorporation was determined via the Cherenkov method and samples with greater than 10^6cpm/μg DNA were used in subsequent shift assays. Binding reactions for SF1 and FOXL2 using nuclear protein extracts were carried out in DNA binding buffer (20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 20% Glycerol, 1 mM DTT, 50 μg/ml poly dI-dC, 2.5 μg/ml salmon sperm DNA, 50 μg/ml BSA) using 1 μl ^{32}P end-

labelled probe and 1 μl nuclear protein extract in a total of 20μl for 30 minutes at room temperature. The incubations were stopped by transfer to ice and 2μl 10x loading dye (0.2M HEPES pH7.9, 0.5M KCl, 10mM EDTA, 0.25% BPB) were added to each sample. 10μl of each sample was subsequently run on 6% acrylamide gels in 0.5x TBE for 4 hours at 100V. The gels were then dried for 30 minutes and analysed by film exposure overnight and for 1 week at -80°C.

Chapter 3

TESCO modulation by ovarian determining genes *in vitro*

3.1 Introduction

The key element in the early gonad for ovarian development is the down-regulation of *Sox9* expression in the supporting cell lineage by 11.5 dpc. If *Sox9* fails to be down-regulated and is instead maintained, XX female-to-male sex reversal occurs. This has been shown in XX human patients with duplication of the chromosomal region containing *SOX9* (17q23-24) and by transgenic misexpression of *Sox9* in XX mice (Huang et al., 1999; Bishop et al., 2000; Vidal et al., 2001). However, it was not known which gene(s) are responsible for the initial down-regulation of *Sox9* expression in XX gonads and the maintenance of this repressed state throughout adult life. Several genes have been proposed to act as ovarian promoting or anti-testis genes and might be involved in the initial down-regulation of *Sox9* and/or in the maintenance of its repression in the XX gonad.

Recently, the 1.3 kb enhancer sequence TESCO was identified as the regulatory

element for sex-specific *Sox9* expression in the XY gonad (Sekido and Lovell-Badge, 2008). Moreover, *in vitro* studies in COS7 cells using a reporter assay approach in which the mouse TESCO element drove the expression of a Luciferase reporter gene, showed that TESCO could not be activated by SRY or SOX9 by themselves. However, it could be activated about 5-fold by SF1, about 10-fold by combination of SF1 and SRY and a robust activation (30-fold) was detected by a combination of SF1 and SOX9 (Sekido and Lovell-Badge, 2008). When the consensus binding sites for both SF1 and SOX proteins were mutated in the TESCO sequence, no activity could be detected in the co-transfection assays, furthermore indicating a synergistic effect between SF1 and SRY/SOX9 in regulating TESCO activity. Studies in mice carrying a CFP reporter transgene under the control of the TESCO element showed that the enhancer activity recapitulates the gonadal expression of *Sox9 in vivo*. While high levels of TESCO activity could only be detected in the presence of endogenous SOX9, some reporter activity was also seen in the absence of SOX9 at 11.5 dpc *in vivo*, indicating that SRY is required for TESCO activation. Furthermore, corresponding to the *in vitro* results, mutation of the SF1 and SOX binding sites abolished TESCO:CFP expression in the transgenic mice (Sekido and Lovell-Badge, 2008). All these data let to the proposal that the TESCO element is necessary to activate and maintain *Sox9* expression in the XY gonad.

On the other hand, it is also possible that the TESCO element plays a role in the regulation of *Sox9* in the XX gonad, either in its initial down-regulation and/or the maintenance of its repression. This hypothesis raises the question whether any of the proposed ovarian determining or anti-testis genes could be involved in the

regulation of TESCO activity in the XX gonad. These genes have been suggested to be important for ovarian development due to their sex-specific expression in XX gonads both during the time of sex determination and in the adult. Thus, the aim of the study presented in this chapter was to analyse whether the ovarian promoting genes could indeed regulate TESCO activity.

To determine whether DAX1, SOX4, β-catenin, FOXL2 and ERα could possibly repress *Sox9* expression via the TESCO element, the TESCO sequence was first analysed for potential binding sites of the proposed transcription factors. Subsequently the individual effect of each factor on the TESCO element was analysed by *in vitro* co-transfection assays.

3.2 Results

3.2.1 Sequence analysis of TESCO

The 1.3 kb TESCO sequence was analysed for putative binding sites of forkhead factors (FOXL2), EREs, nuclear receptors (DAX1) and LEF/TCF factors (as effectors of Wnt4-signalling) using the MatInspector program (Quandt et al., 1995).

The search revealed two possible forkhead factor binding sites, three ERE half sites, two LEF/TCF binding sites and three nuclear receptor sites (Figure 3.1). In addition to the sites identified in this study, the TESCO sequence also contains six SF1-specific binding sites and three sites for SOX proteins (Sekido and Lovell-Badge, 2008). From the Ensemble database, the TESCO sequences were compiled for mouse (ENSMUSG00000000567), rat (ENSRNOG00000002607), human (ENSG00000125398) and dog (ENSCAFG00000004374) and then analysed by the NCBI-BLAST software. A comparison between those species showed high conservation for most of the predicted binding sites (see Appendix 8.3), suggesting a potential role for these sites in *Sox9* regulation.

To test if FOXL2, ERα, DAX1, SOX4 or the β-catenin/LEF/TCF complex could indeed exert a repressive effect on the TESCO element, *in vitro* co-transfection assays were carried out.

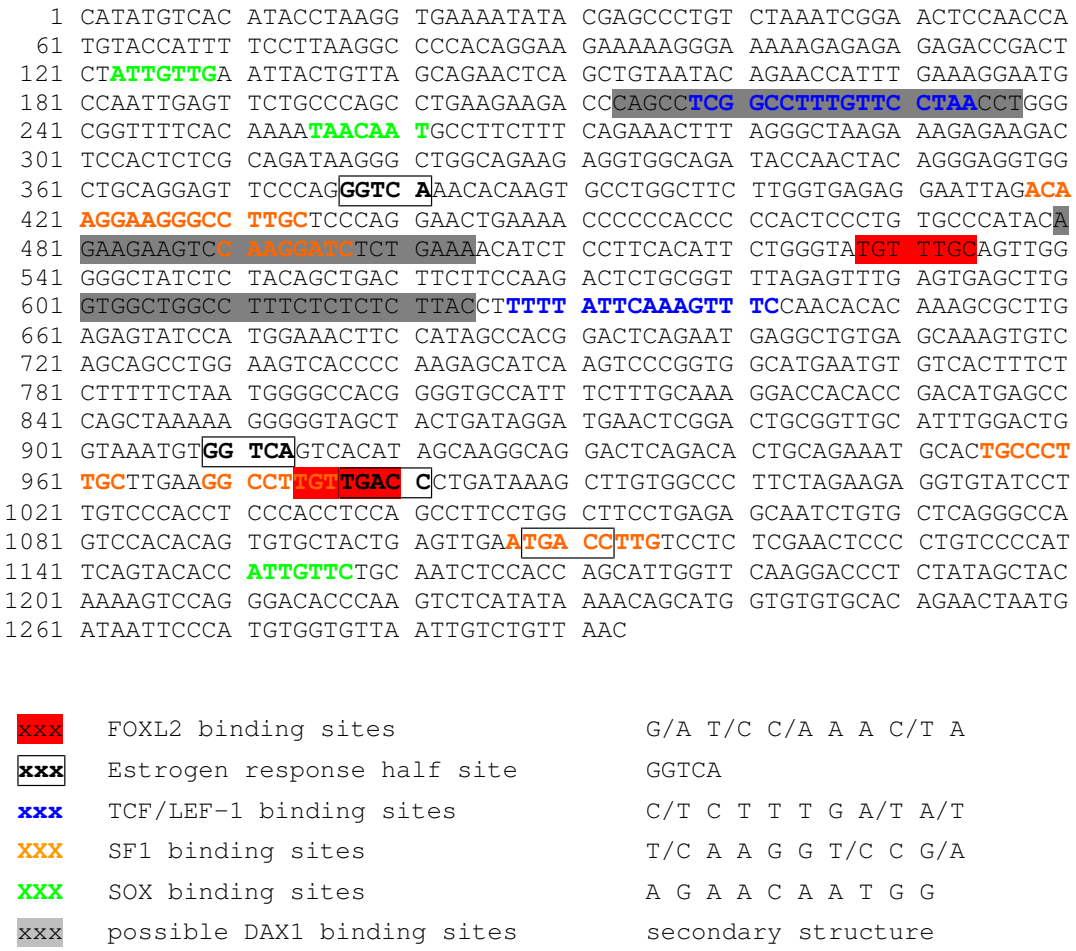


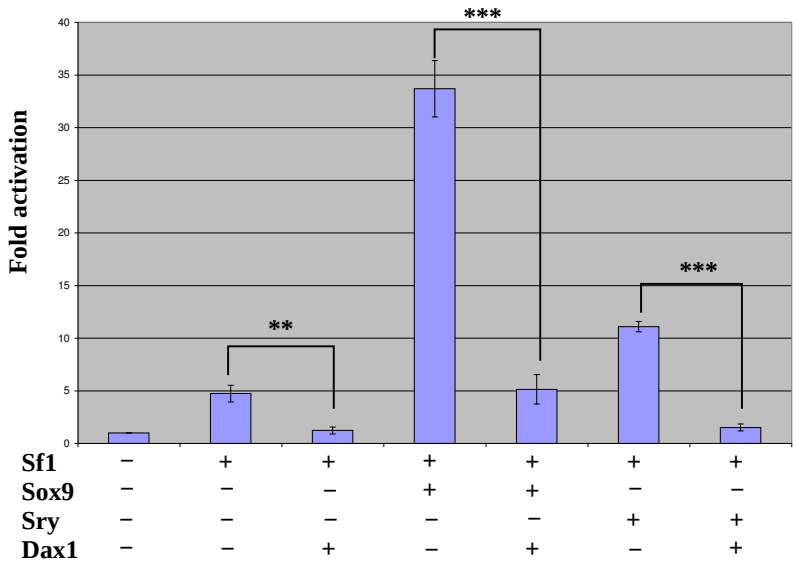
Figure 3.1: **Sequence analysis of TESCO.**
The TESCO sequence contains several binding sites for forkhead factors (FOXL2), EREs, nuclear receptor sites (DAX1) and binding sites for LEF/TCF factors, as well as SF1 and SOX binding sites.

3.2.2 Repression of TESCO *in vitro*

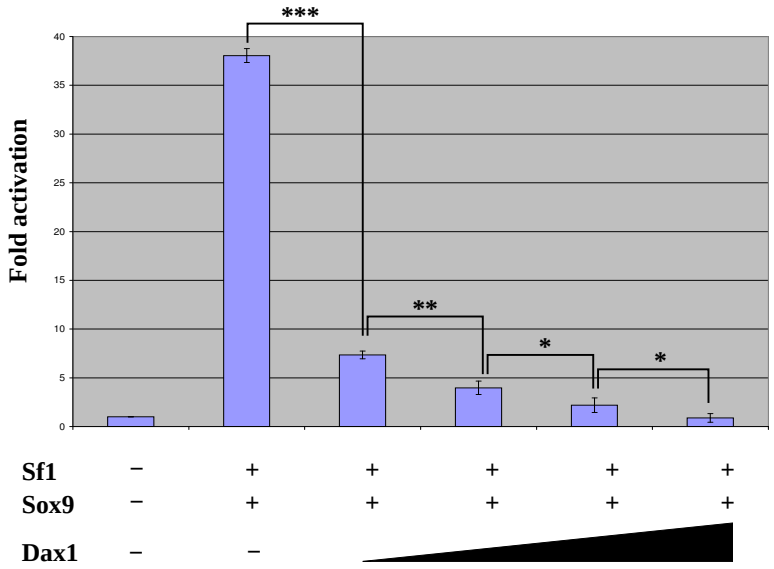
To investigate the possible repressive effects of DAX1, SOX4, WNT4, FOXL2 and ERα, each of the genes was cloned into the same expression vector used for SF1 and SRY. Each construct was then expressed in COS7 cells together with the TESCO reporter construct and combinations of SF1, SF1 + SOX9 and SF1 + SRY, respectively. Dosage-dependent effects were analysed by co-transfection of different amounts of the candidate gene upon TESCO activation by SF1 + SOX9.

Addition of DAX1 resulted in 74% reduction of TESCO activation when activated with SF1, 85% when activated with SF1 + SOX9 and 86% when activated with SF1 + SRY (Figure 3.2a). Increasing amounts of DAX1 showed a dosage-dependent reduction of TESCO activity down to background levels (Figure 3.2b).

Addition of SOX4 did not have any significant effect on TESCO activation by SF1, but co-transfection of SOX4 with SF1 + SRY or SF1 + SOX9 resulted in a reduction of around 10% (Figure 3.3a). Increasing amounts of SOX4 correlated with a greater repression of TESCO activity, up to a reduction of about 60% of the original activation by SF1 + SOX9 (Figure 3.3b).

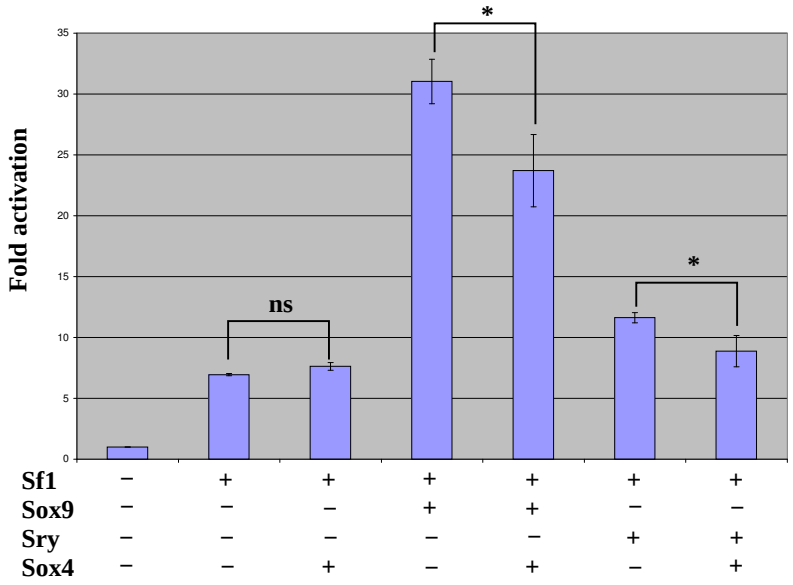


(a)

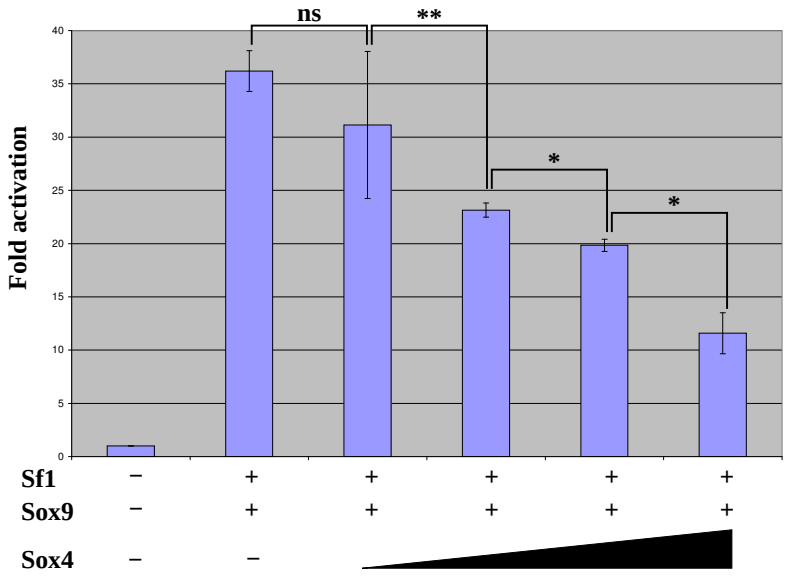


(b)

Figure 3.2: **Repression of TESCO by DAX1 *in vitro*.**
SF1, SF1 + SOX9 or SF1 + SRY mediated TESCO activation was robustly repressed *in vitro* by co-transfection of DAX1 (a). This effect was dosage-dependent as higher amounts of *Dax1* resulted in a greater repression of TESCO (b). Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



(a)

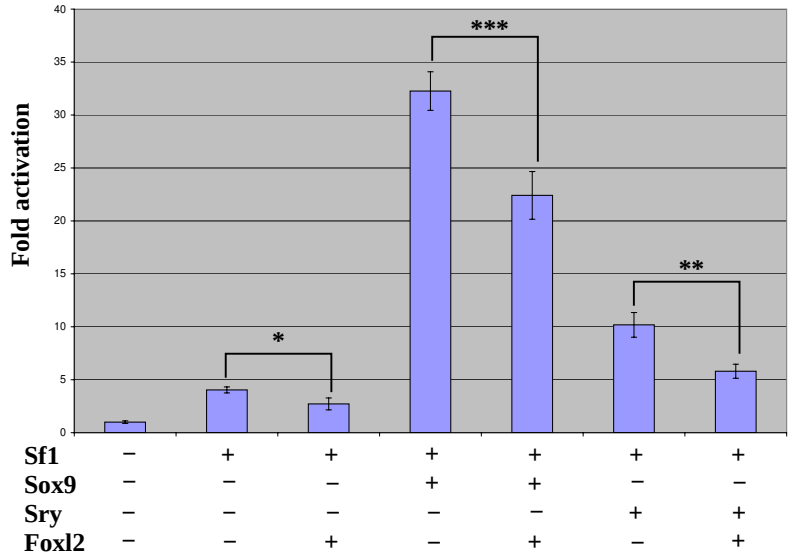


(b)

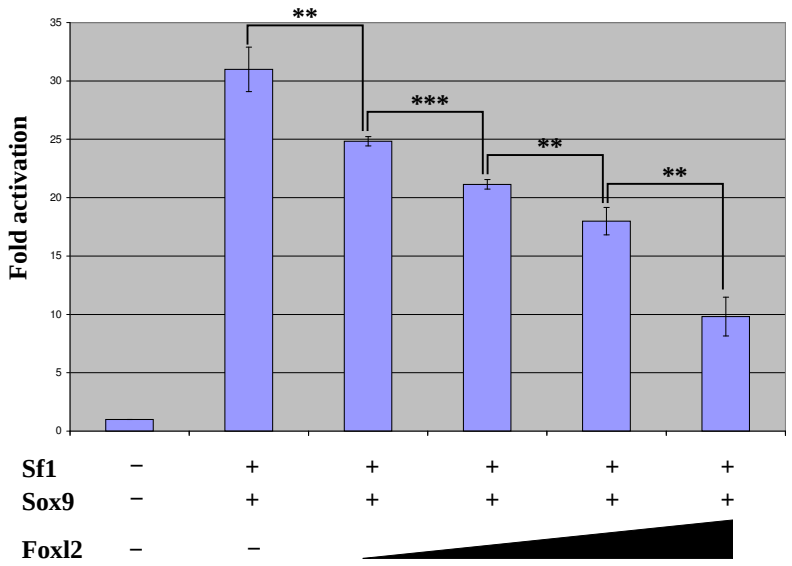
Figure 3.3: **Repression of TESCO by SOX4 *in vitro*.**
SOX4 did not have any significant effect on the activation of TESCO by SF1 alone, but in combination with SF1 + SOX9 or SF1 + SRY resulted in a minor repression of TESCO activation (a). Increasing amounts of SOX4 revealed a dosage-dependent repression effect on SF1 + SOX9 mediated TESCO activation (b). Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with * $p < 0.05$, ** $p < 0.01$, ns = not significant.

The co-transfection assays with FOXL2 were first done using an EGFP-Foxl2 N-terminal fusion plasmid (gift from M. Treier). These assays showed a reduction of TESCO activity by 32% when activated with SF1, by 35% when activated with SF1 + SOX9 and a reduction by 43% when activated with SF1 + SRY (Figure 3.4a). This effect was dosage-dependent as higher amounts of FOXL2 resulted in greater TESCO repression, up to a reduction of about 72% of the original activation by SF1 + SOX9 (Figure 3.4b). However, because EGFP can dimerise and this might compromise the activity of the linked FOXL2, the co-transfection assays were repeated using a different construct containing only the *Foxl2* gene. The same results were obtained: reduction of TESCO after activation by SF1, SF1 + SOX9 or SF1 + SRY and a dosage-dependent repression effect (data not shown).

To investigate a possible interaction between FOXL2 and estrogen receptors during sex determination, the effect of ERα on TESCO activity was analysed on its own and in combination with FOXL2. ERα alone showed no significant effect on TESCO activation by SF1 + SOX9. However, when ERα was co-transfected with FOXL2 it enhanced the FOXL2-mediated repression of TESCO activation, down to around 10% of the original activation by SF1 + SOX9. This synergistic repression effect of ERα seemed to be independent of the amount used (Figure 3.5).



(a)



(b)

Figure 3.4: **Repression of TESCO by FOXL2 *in vitro*.**
TESCO activation was repressed *in vitro* by co-transfection of FOXL2 to SF1, SF1 + SOX9 or SF1 + SRY, respectively (a). This effect was dosage-dependent as higher amounts of *Foxl2* resulted in a greater repression of TESCO (b). Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

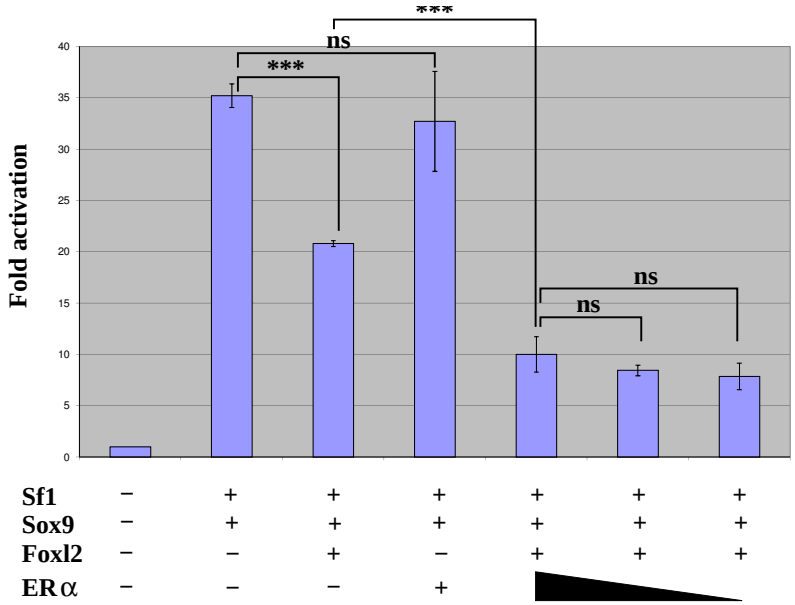
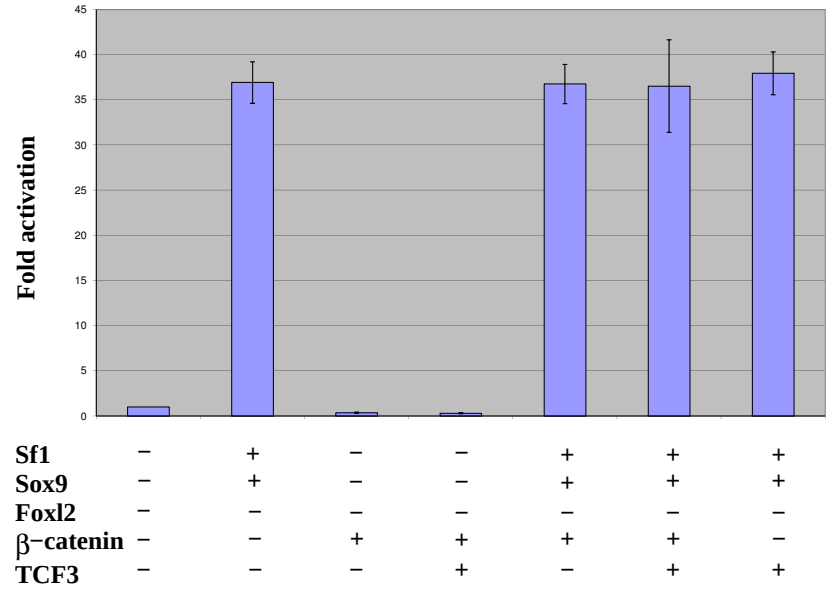


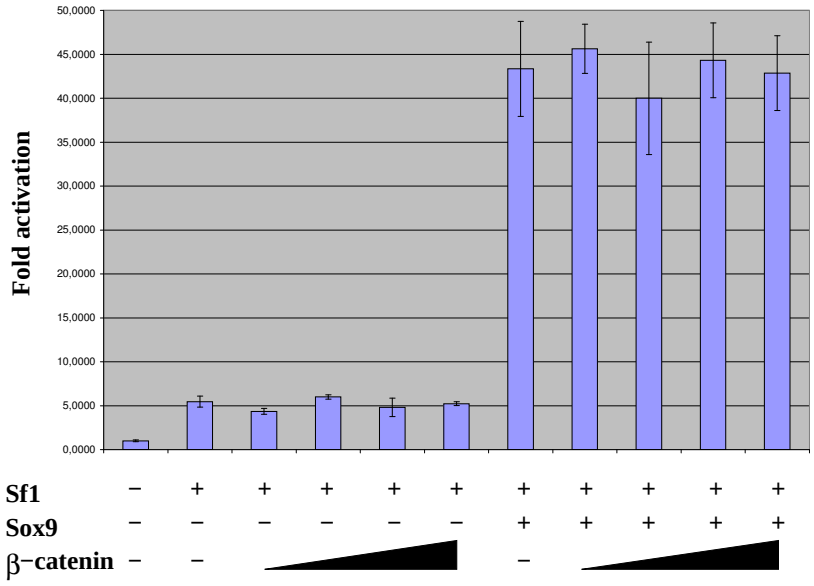
Figure 3.5: **Repression of TESCO by ERα *in vitro*.**
ERα on its own did not have any effect on SF1 + SOX9 mediated activation of TESCO, but it enhanced the repression effect mediated by FOXL2. This enhanced repression was independent of the amount of ERα used. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with *** $p < 0.001$, ns = not significant.

Next, the effect of WNT signalling on TESCO activation was assessed. WNT4 itself did not activate the WNT-reporter plasmid TOPflash (Molenaar et al., 1996) in COS7 cells (data not shown), which suggests that the WNT4 signalling pathway is not active in this cell line. Thus, the effectors of the signalling pathway, such as β-catenin and LEF1, TCF1, TCF3, TCF4 were used in the co-transfection assays to test the implication of WNT signalling on TESCO activity. As the β-catenin/LEF/TCF complex usually acts as a transcriptional activator, it was theoretically possible that this complex could activate TESCO on its own or enhance the activation mediated by SF1 or SF1 + SOX9. However, co-transfection assays with β-catenin alone or in combination with any LEF/TCF factor did not activate TESCO in the absence of SF1 + Sox9 (Figure 3.6a). Moreover, TESCO activation by SF1 + Sox9 was not significantly altered by addition of β-catenin alone, any of the LEF/TCF factors on their own or by combinations of β-catenin and any of the LEF/TCF factors (Figure 3.6a). For both assays, the results are shown for TCF3 though the same results were obtained for TCF1, TCF4 and LEF1 (data not shown). Even using increasing amounts of β-catenin did not result in any effect on TESCO activation by SF1 or SF1 + SOX9 (Figure 3.6b).

Next, the possible enhancement of forkhead transcriptional activity by interaction with β-catenin was analysed. As FOXL2 represses TESCO activation, the presence of β-catenin could result in a stronger repression. However, co-transfection assays showed that addition of β-catenin did not have any effect on the FOXL2-mediated repression of TESCO activity (Figure 3.7).



(a)



(b)

Figure 3.6: **Effect of β-catenin and LEF/TCFs on TESCO activation *in vitro*.**
β-catenin did not activate TESCO on its own or with TCF3. Activation of TESCO by SF1 + SOX9 was not affected by addition of β-catenin, TCF3 or both (a). Increasing amounts of β-catenin did not result in any effect on either SF1 or SF1 + SOX9 mediated activation of TESCO (b). Assays were performed in triplicate and bars indicate the standard deviation of three biological replicates.

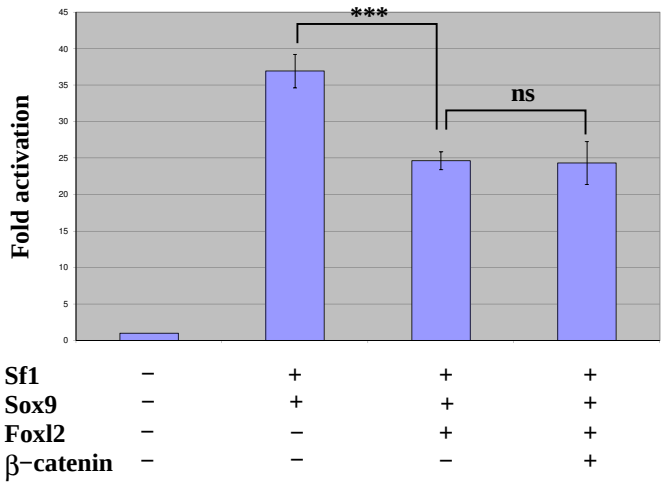


Figure 3.7: **Effect of β-catenin on FOXL2 mediated repression of TESCO *in vitro*.**
β-catenin did not have any significant effect on FOXL2 mediated repression of TESCO. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with *** $p < 0.001$, ns = not significant.

3.3 Conclusions

In the early XX gonad, *Sox9* expression is down-regulated in the supporting cell lineage at 11.5 dpc and this repression is maintained throughout life. Although a number of genes have been proposed as ovarian promoting or anti-testis genes, such as *Wnt4*, *β-catenin*, *Dax1*, *Sox4*, *Foxl2* and ERα, it is not known if any of these genes has a primary role in the repression of *Sox9*. In the XY gonad, *Sox9* expression is regulated via the TESCO enhancer element (Sekido and Lovell-Badge, 2008), it is possible that the down-regulation in the XX gonad is mediated via this element as well. To determine whether the proposed ovarian genes have the potential to repress *Sox9* expression by modulating TESCO activity, *in vitro* co-transfection assays were performed.

In the WNT signalling pathway, LEF/TCFs are the executive factors in Wnt canonical pathways, which lead, in association with β-catenin, to the activation of downstream target genes. On the other hand, it has been shown that LEF/TCF proteins can physically interact with co-repressors, such as Groucho and CtBP and thereby lead to the repression of their target genes (Roose et al., 1998). Moreover, TCF3 has been demonstrated to act as a repressor in the absence of stabilised β-catenin (Merrill et al., 2004). Thus, there are several different possibilities as to how β-catenin and LEF/TCF factors could be involved in *Sox9* regulation: β-catenin in combination with any LEF/TCF factor could act as an activator of *Sox9* expression via the TESCO element or TCF3 could act as a repressor in the absence of β-catenin. However, the co-transfection assays showed that β-catenin does not affect TESCO on its own or in combination

with any LEF/TCF factor, indicating that these effectors are not contributing to the activation of the TESCO element. Moreover, SF1 + SOX9 mediated activation of TESCO was not affected by TCF3, arguing against the hypothesis that TCF3 might at as a repressor of *Sox9* in the absence of β-catenin. It has been reported that forkhead factors can compete with TCFs for the interaction with β-catenin. The formation of a forkhead/β-catenin complex has been shown to enhance forkhead transcriptional activity (Hoogeboom et al., 2008). However, the results obtained in this thesis show that addition of β-catenin did not significantly affect FOXL2 mediated repression of TESCO activity. Taken together, none of the tested effectors of the WNT signalling pathway had any significant effect on TESCO activation in the *in vitro* co-transfection assays. This could imply that WNT signalling is not affecting *Sox9* transcription or that it is not acting simply through the TESCO element but possibly via other regulatory elements. However, it is also possible that necessary co-factors involved in the signalling process or in SOX9-β-catenin degradation (like Groucho or CtBP) are missing in this type of *in vitro* assay system and therefore no effect can be seen due to the limitations of the system. Another intriguing possibility is that WNT-signalling could affect *Sox9* expression only indirectly, e.g. via DAX1. It has already been demonstrated that *Dax1* expression is significantly decreased in XX *Wnt4*-deficient mice and that *Dax1* transcription is activated by β-catenin (in cooperation with SF1), indicating that *Dax1* is a downstream target gene of *Wnt4*. Therefore, it is possible that *Wnt4* signalling might act solely through β-catenin mediated activation of *Dax1* (Mizusaki et al., 2003) in sex determination.

All other candidate genes tested in the *in vitro* co-transfection assays in this

chapter did have an effect on TESCO expression. *Dax1* was able to repress TESCO activation in a dosage-dependent manner. The repression effect of *Dax1* was the strongest of all genes analysed. It is already known that DAX1 can form heterodimers with SF1, which results in a decrease of SF1 activity (Ito et al., 1997; Crawford et al., 1998). Since SF1 is required for TESCO activation, a reduction in SF1 activity in the presence of DAX1 could cause the observed repression of TESCO. However, the detailed mechanism as to how DAX1 represses TESCO activation, whether by direct binding to the TESCO sequence or by interaction with SF1, remains to be investigated.

SOX4 also repressed TESCO activation, though it had the weakest repressor effect of all genes analysed. Interestingly, SOX4 did not have a repressive effect on TESCO activation mediated by SF1 alone, but was able to repress both SF1 + SOX9 and SF1 + SRY mediated TESCO activation. The TESCO sequence contains multiple SOX binding sites, which are crucial for its activation by SRY and SOX9. SOX4 contains the same, highly conserved, DNA binding domain (HMG-box) as other SOX proteins and probably binds to the same sites in the DNA. Thus, it is possible that SOX4 competes with SOX9 and SRY for binding to the TESCO enhancer sequence. It could be speculated that SOX4 and SOX9, as members of different groups of SOX proteins, exhibit different effects on the TESCO element: SOX9 (belonging to the SOXE group) activates TESCO while SOX4 (belonging to the SOXC group) might act as a repressor or just reduces the amount of SOX9 bound to TESCO.

FOXL2 was able to repress TESCO activity in a dosage-dependent manner,

whereas ERα had no effect on TESCO activation. Interestingly, the repressive effect of FOXL2 was even more severe in the presence of ERα, suggesting a synergistic effect of the two proteins on TESCO activation. However, this synergistic repressive effect seems to be independent of the amount of ERα available.

In summary, the proposed ovarian promoting genes *Dax1*, *Sox4* and *Foxl2* (on its own or in combination with *ER*α) are able to repress the activation of the TESCO element *in vitro*. This suggests that they might indeed be involved in the regulation of *Sox9* expression *in vivo* via the TESCO element. Moreover, the result that FOXL2 can repress TESCO activity provides the first direct link between FOXL2 and *Sox9* regulation. To gain more insight into FOXL2 mediated repression of TESCO, possible mechanisms of this regulatory effect were analysed *in vitro*.

Chapter 4

Possible mechanisms of TESCO modulation by FOXL2

4.1 Introduction

FOXL2 is able to repress *Sox9* expression via the TESCO enhancer element *in vitro*. However, it is important to further determine how FOXL2 interacts with TESCO and how exactly the repression is mediated.

FOXL2 is a member of the forkhead gene family of transcription factors (Crisponi et al., 2001) and members of this family are characterised by a DNA binding domain consisting of a helix-turn-helix core element of three α-helices flanked by two loops or wings (Figure 4.1). The recognition Helix 3 interacts with the major groove of DNA and Wing 2 with the minor groove. The sequence of this forkhead domain (FHD) is highly conserved, indicating a similar 3D structure and DNA recognition within the forkhead factor family, whereas regions outside the forkhead domain are poorly conserved between family members (Carlsson and Mahlapuu, 2002). Forkhead proteins bind DNA as monomers to the consensus

binding site (A/G)(C/T)(A/C)AA(A/T)A (Kaufmann et al., 1995) and in doing so bend the DNA up to an angle of 80-90°(Pierrou et al., 1994). This property is unusual as most transcription factors do not induce such a distortion in DNA. However, the HMG boxes of SOX proteins are also known to bend the DNA up to 90°by binding to the minor groove (Sekido and Lovell-Badge, 2008). The flanking regions on both sides of the consensus sequence are important for the protein binding affinity to the DNA. Forkhead factors have been shown to act as both transcriptional activators and repressors (Carlsson and Mahlapuu, 2002) and can be found in a variety of vertebrate and non-vertebrate species indicating a conserved mechanism of action (Mazet et al., 2003).

FOXL2 is a nuclear protein and contains the arginine/lysine-rich sequence of the classical nuclear localisation signal (NLS) at the C-terminus of the forkhead domain (Figure 4.1). In addition, FOXL2 also comprises a non-conventional NLS (KGNYWTLDPACEDMFEKGNY) just prior to the classical motif (Moumné et al., 2008). The C-terminal part of the FOXL2 protein contains two polyA and one polyP stretch (Figure 4.1). About 30% of all reported human FOXL2 mutations are attributed to expansion of the polyA tract from the normal 14 alanines to up to 24 (Moumné et al., 2008). *In vitro* analyses have shown that expansion of the polyA stretch results in mislocalisation and cytoplasmic aggregation of the protein. FOXL2 is normally exclusively localised in the nucleus and therefore cytoplasmic aggregation results in loss of its activity. On the other hand, a patient with primary amenorrhea carrying a 30 bp deletion in the *FOXL2* ORF resulting in a reduction of the polyA tract down to 4 alanines, was able to naturally deliver two healthy babies (Harris et al., 2002; Gersak et al., 2004).

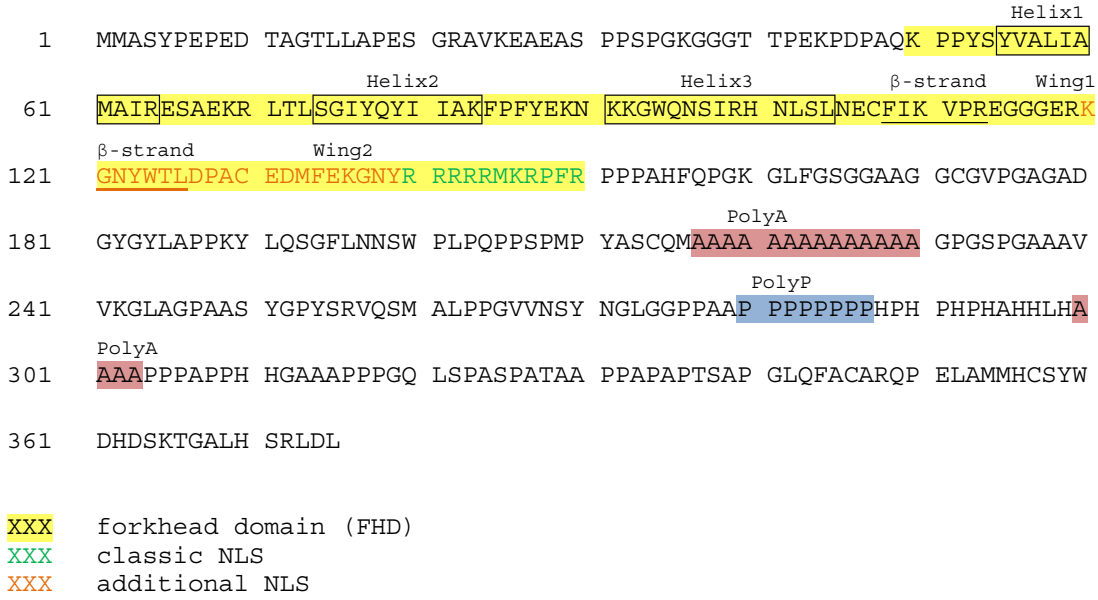


Figure 4.1: **The FOXL2 protein.**
The FOXL2 protein consists of 375 amino acids, the forkhead domain (FHD) ranges from amino acid 50 - 150 and includes three helices, two β-strands and two loop (or wing) structures. FOXL2 contains a classical and an additional nuclear localisation sequence (NLS) at the C-terminal end of the FHD. Outside the FHD are two poly-A stretches and one poly-P stretch.

Only a few target genes for FOXL2 have been described so far. The first direct target to be identified was the promoter of murine gonadotrophin releasing hormone receptor (GnRHR). *In vitro* assays demonstrated that FOXL2 together with SMAD3/4 activated the GnRHR activating element (GRAS) (Ellsworth et al., 2003). Another FOXL2 target gene is *Cyp19* which encodes aromatase, the enzyme that converts androgens into estrogens. In the goat, FOXL2 and CYP19 co-localise in somatic cells of the XX gonad (Pannetier et al., 2006a) and *Cyp19* expression is drastically decreased upon FOXL2 loss in XX foetuses carrying the PIS mutation (Pailhoux et al., 2001). Moreover, FOXL2 was able to bind and activate the goat *Cyp19* promoter *in vitro*, suggesting that it is involved in the regulation of estrogen production in the female gonad (Pannetier et al.,

2006b). FOXL2 could also directly bind to the promoter of human steroidogenic acute regulatory gene (*StAR*), which controls the rate-limiting step in steroidogenesis, and repress its transcription (Pisarska et al., 2004). *StAR* is expressed in differentiated granulosa cells and repression by FOXL2 is thought to prevent accelerated follicular development mediated by an increase of *StAR* activity, which would ultimately lead to premature ovarian failure (POF). *FOXL2* transcripts with a truncation of the C-terminal domain resulting in the loss of the polyalanine tracts, failed to repress *StAR* activity *in vitro* (Pisarska et al., 2004). Recently it has been shown that the activity of FOXL2 as a transcriptional repressor of the *StAR* gene depends on sumoylation. Indeed, FOXL2 and the E2-conjugating enzyme Ubc9 are co-expressed in granulosa cells of small and medium follicles of the developing ovary and FOXL2 is sumoylated at lysine-25 via Ubc9-mediated sumoylation (Kuo et al., 2009). FOXL2 was also able to repress the rat *Dmrt1* promoter in *in vitro* co-transfection assays. The *Dmrt1* promoter contains several forkhead factor binding sites and removal of these sites, by shortening of the promoter sequence, resulted in the loss of repression mediated by FOXL2 (Lei et al., 2009). A bioinformatic screen using the human steroidogenic granulosa-like cell line KGN, identified new potential FOXL2 target genes, which suggest the involvement of FOXL2 the regulation of a number of different genes, including immunomodulators (e.g. IFNB1, IL12A), transcription factors (e.g. NFATC2, NR5a2) and regulators of apoptosis (e.g. BCL2A1, IER3) (Batista et al., 2007).

Forkhead factors have been described to act as co-repressors on their target genes by directly interacting with other proteins: for example, FOXG1 interacts with androgen receptor (AR) and thereby acts as an AR co-repressor (Obendorf et al.,

2007) and FOXP1 interacts with SMRT to repress the differentiation gene *c-fms* in monocytes (Jepsen et al., 2008). FOXL2 has been found to directly interact with few proteins so far. It has been shown to interact with the dead box protein DP103 resulting in an increase in apoptosis *in vitro* (Lee et al., 2005). Moreover, FOXL2 interacts with SMAD3 to regulate the transcription of Follistatin *in vitro* (Blount et al., 2009) and in tilapia, FOXL2 and SF1 interact to activate *Cyp19* gene expression (Wang et al., 2007). Direct interaction has also been demonstrated between FOXL2 and ERα in co-immunoprecipitation experiments *in vitro* (Uhlenhaut et al., 2009; Kim et al., 2009).

The results presented so far, have shown that FOXL2 can repress TESCO activity *in vitro*. The TESCO element contains forkhead factor binding sites suggesting that FOXL2 could directly bind to the TESCO element. Indeed, chromatin immunoprecipitation assays have demonstrated that FOXL2 can bind to the TESCO enhancer element of *Sox9* (Uhlenhaut et al., 2009). In this chapter, possible mechanisms of how FOXL2 is mediating its repressive effect on TESCO was analysed in more detail *in vitro*.

4.2 Results

4.2.1 Analyses of mutations in TESCO binding sites

The TESCO element contains two highly conserved consensus forkhead factor binding (FOX) sites (Figure 3.1) through which the repression effect of FOXL2 could be mediated. Each of these binding sites was mutated individually as well as in combination, by replacing the FOX sites with SacI restriction sites, to determine their importance for FOXL2 binding. The mutated TESCO sequences were then cloned into the luciferase vector and their activities were analysed in co-transfection assays with SF1 + SOX9 and SF1 + SOX9 + FOXL2 using COS7 cells, respectively. Neither individual nor combined mutations of those sites resulted in a statistically significant loss of FOXL2 mediated repression of TESCO activity (Figure 4.2).

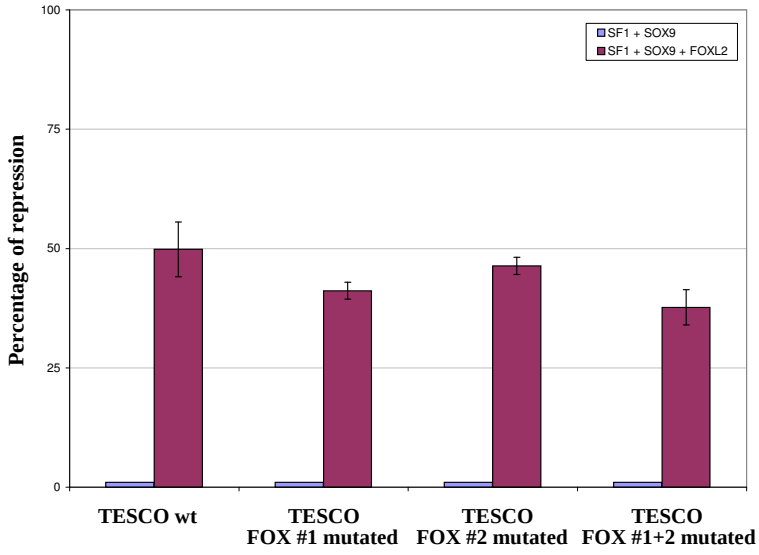


Figure 4.2: **Effect of FOXL2 on TESCO with 2 mutated FOX sites.** TESCO activation by SF1 + SOX9 could be repressed by addition of FOXL2, even after mutation of the highly conserved forkhead factor binding sites (#1: bp 528-534 and #2: bp 974-980) either individually or simultaneously. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

It was possible that FOXL2 could still repress TESCO activity after mutation of these two consensus binding sites because the TESCO element might contain other forkhead factor binding sites which had not been picked up by the initial search with the MatInspector program. Thus, the TESCO sequence was searched manually for the core sequence of forkhead factor binding sites (C/A)AA(C/T)A. The search revealed 10 possible binding sites, including the two sites found in the original search, evenly distributed over the TESCO sequence (Figure 4.3).

To determine the importance of these sites, several mutation constructs of the TESCO sequence were analysed in co-transfection assays. These constructs contained 200 - 250 bp deletions in different parts of the TESCO sequence: deletion of bp 72 to 335 including two possible FOX sites (ΔC4), deletion of bp 354 to 548 including three possible FOX sites (ΔC5), deletion of bp 564 to 829 including one possible FOX site (ΔC6), deletion of bp 847 to 1054 including one possible FOX site (ΔC7). Another construct contained a shorter version of TESCO, lacking 200bp at the 3'-end which included one possible FOX site (1.0 kb). For a detailed sequence map see Appendix Figure 8.4. All of the constructs (except ΔC6) also contained established binding sites for SF1 and/or SOX9. Due to the reduced number of activation sites, the overall activation potential of these deletion constructs was diminished when co-transfected with SF1 + SOX9. However, repression of TESCO activity upon addition of FOXL2 was found in all these constructs. A slight reduction in repression ability of FOXL2 could be seen in the assays with the ΔC6 and ΔC7 constructs, but these differences were not statistically significant (Figure 4.4).

```
   1 CATATGTCAC ATACCTAAGG TGAAAATATA CGAGCCCTGT CTAAATCGGA ACTCCAACCA
  61 TGTACCATTT TCCTTAAGGC CCCACAGGAA GAAAAAGGGA AAAAGAGAGA GAGACCGACT
 121 CTATTGTTGA ATTACTGTTA GCAGAACTCA GCTGTAATAC AGAACCATTT GAAAGGAATG
 181 CCAATTGAGT TCTGCCCAGC CTGAAGAAGA CCCAGCCTCG GCCTTTGTTC CTAACCTGGG
 241 CGGTTTTCAC AAAATAACAA TGCCTTCTTT CAGAAACTTT AGGGCTAAGA AAGAGAAGAC
 301 TCCACTCTCG CAGATAAGGG CTGGCAGAAG AGGTGGCAGA TACCAACTAC AGGGAGGTGG
 361 CTGCAGGAGT TCCCAGGGTC AAACACAAGT GCCTGGCTTC TTGGTGAGAG GAATTAGACA
 421 AGGAAGGGCC TTGCTCCCAG GAACTGAAAA CCCCCCACCC CCACTCCCTG TGCCCATACA
 481 GAAGAAGTCC AAGGATCTCT GAAAACATCT CCTTCACATT CTGGGTATGT TTGCAGTTGG
 541 GGGCTATCTC TACAGCTGAC TTCTTCCAAG ACTCTGCGGT TTAGAGTTTG AGTGAGCTTG
 601 GTGGCTGGCC TTTCTCTCTC TTACCTTTTT ATTCAAAGTT TCCAACACAC AAAGCGCTTG
 661 AGAGTATCCA TGGAAACTTC CATAGCCACG GACTCAGAAT GAGGCTGTGA GCAAAGTGTC
 721 AGCAGCCTGG AAGTCACCCC AAGAGCATCA AGTCCCGGTG GCATGAATGT GTCACTTTCT
 781 CTTTTTCTAA TGGGGCCACG GGGTGCCATT TCTTTGCAAA GGACCACACC GACATGAGCC
 841 CAGCTAAAAA GGGGGTAGCT ACTGATAGGA TGAACTCGGA CTGCGGTTGC ATTTGGACTG
 901 GTAAATGTGG TCAGTCACAT AGCAAGGCAG GACTCAGACA CTGCAGAAAT GCACTGCCCT
 961 TGCTTGAAGG CCTTGTTGAC CCTGATAAAG CTTGTGGCCC TTCTAGAAGA GGTGTATCCT
1021 TGTCCCACCT CCCACCTCCA GCCTTCCTGG CTTCCTGAGA GCAATCTGTG CTCAGGGCCA
1081 GTCCACACAG TGTGCTACTG AGTTGAATGA CCTTGTCCTC TCGAACTCCC CTGTCCCCAT
1141 TCAGTACACC ATTGTTCTGC AATCTCCACC AGCATTGGTT CAAGGACCCT CTATAGCTAC
1201 AAAAGTCCAG GGACACCCAA GTCTCATATA AAACAGCATG GTGTGTGCAC AGAACTAATG
1261 ATAATTCCCA TGTGGTGTTA ATTGTCTGTT AAC

XXX   SF1 binding sites                  T/C A A G G T/C C G/A
XXX   SOX binding sites                  A G A A C A A T G G
XXX   FOXL2 core binding site            G/A T/C C/A A A C/T A
```

Figure 4.3: **Additional consensus FOX sites in the TESCO sequence.** The TESCO sequence was manually searched for the core sequence of forkhead factor binding sites (C/A)AA(C/T)A, which revealed 10 possible FOX sites evenly distributed over the TESCO sequence.

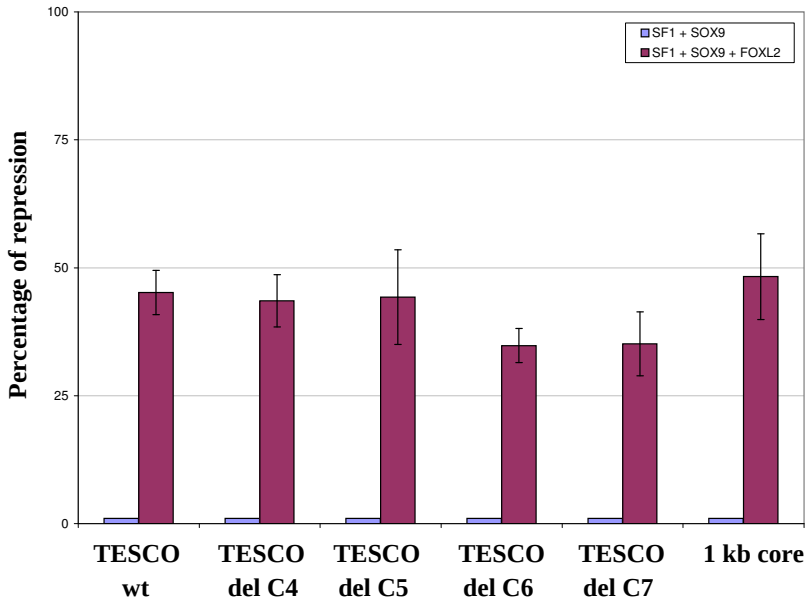


Figure 4.4: **Repression effect of FOXL2 on TESCO deletion constructs.** Five constructs with deletions of different parts of TESCO were analysed in co-transfection assays. Addition of FOXL2 after activation by SF1 + SOX9 resulted in a repression similar to the wild-type TESCO construct. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

Next, the all ten putative FOX sites in the TESCO element were mutated simultaneously by replacement of the essential central AA motif with a GC motif. The mutated TESCO sequence was then cloned into the luciferase vector and analysed *in vitro* as described before. The construct was activated by SF1 + SOX9 in co-transfection assays and addition of FOXL2 still resulted in repression of the TESCO activity (Figure 4.5). The combined mutation of all FOX sites and all estrogen response elements (EREs) still resulted in repression of TESCO activity by FOXL2, but interestingly the previously observed synergistic repression effect of ERα together with FOXL2 was abolished (Figure 4.6).

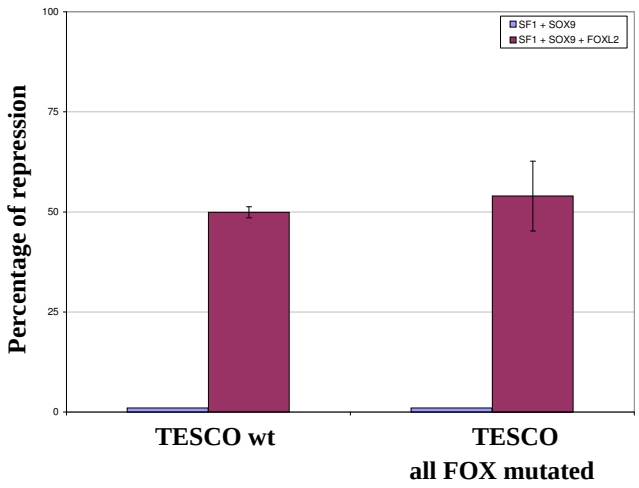


Figure 4.5: **Repression effect of FOXL2 on TESCO with mutations in all consensus FOX sites.**
The ten possible consensus FOX sites found in the TESCO sequence were mutated and the construct used in co-transfection assays. Addition of FOXL2 after activation by SF1 + SOX9 resulted in a repression of the mutant TESCO similar to the wild-type TESCO element. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

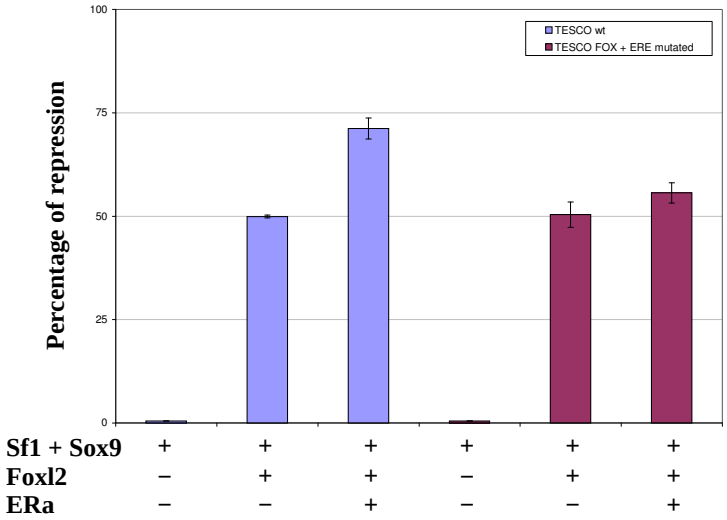


Figure 4.6: **Repression effect of FOXL2 + ERα on TESCO with all FOX/ERE sites mutated.**
All FOX and ERE sites found in the TESCO element were mutated and the construct used in co-transfection assays. Addition of FOXL2 after activation by SF1 + SOX9 resulted in a repression of the mutant TESCO similar to the wild-type TESCO element. However, the synergistical effect of ERα in enhancing FOXL2 mediated repression was lost. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

In all the mutation constructs analysed in the above co-transfection assays, addition of FOXL2 resulted in a similar level of repression of TESCO activity. Thus, it could be possible that the repression effect seen by FOXL2 might be due to a non-specific effect of FOXL2 on the luciferase vector backbone. To rule out this possibility, increasing amounts of the original luciferase vector (without the TESCO sequence) were added in co-transfection assays to the wild-type TESCO-luciferase construct which was activated by SF1 + SOX9 and repressed by addition of FOXL2, as described before. If FOXL2 was indeed binding to cryptic binding sites in the vector backbone, excess amounts of Luciferase vector would deplete the available FOXL2, thereby reducing the amount of FOXL2 accessible to repress TESCO activity. However, even with increasing amounts of Luciferase vector, no significant decrease in the FOXL2-mediated repression of TESCO activity was found (Figure 4.7). It might be worth noting that, due to the large excess of the original luciferase vector used in this assays, the cumulative amount of DNA was more than double the amount used in the assays before, which might interfere with the transfection efficiency.

Taken together, all these results suggest that the identified FOX binding sites within the TESCO enhancer are not crucial for FOXL2 to repress TESCO activity. It is possible that the TESCO element contains additional forkhead factor binding sites, through which the repression effect of FOXL2 is mediated and which might differ from the known consensus binding site. Another possible explanation is that the repression effect of FOXL2 is not mediated through binding to the TESCO sequence but through a different mechanism, e.g. by acting as a co-repressor in association with another protein.

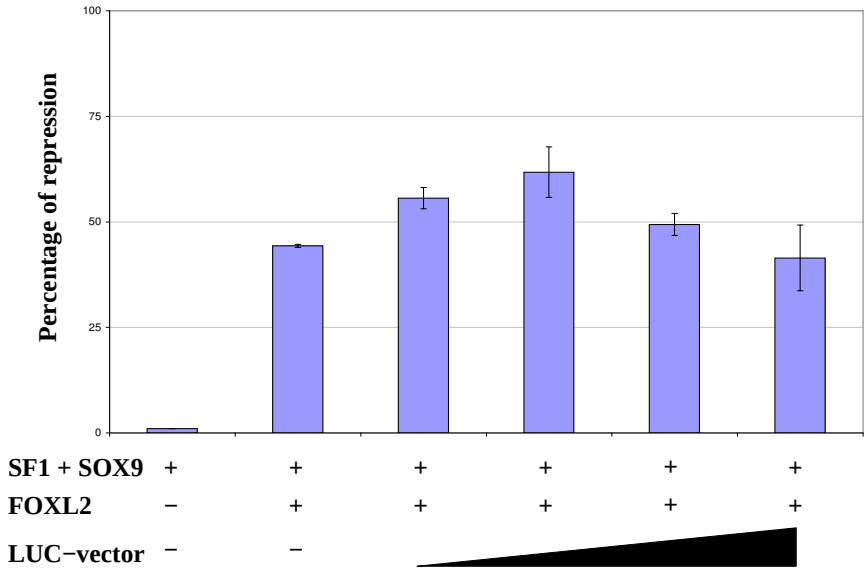


Figure 4.7: **Effect of excess amounts of vector on FOXL2 mediated repression of TESCO.**
Increasing amounts of empty Luciferase vector (not containing the TESCO sequence) did not result in a dosage-dependent loss of FOXL2 mediated repression. This shows that FOXL2 is not binding to cryptic binding sites in the backbone of the vector. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

4.2.2 Analyses of interactions between FOXL2 and SF1

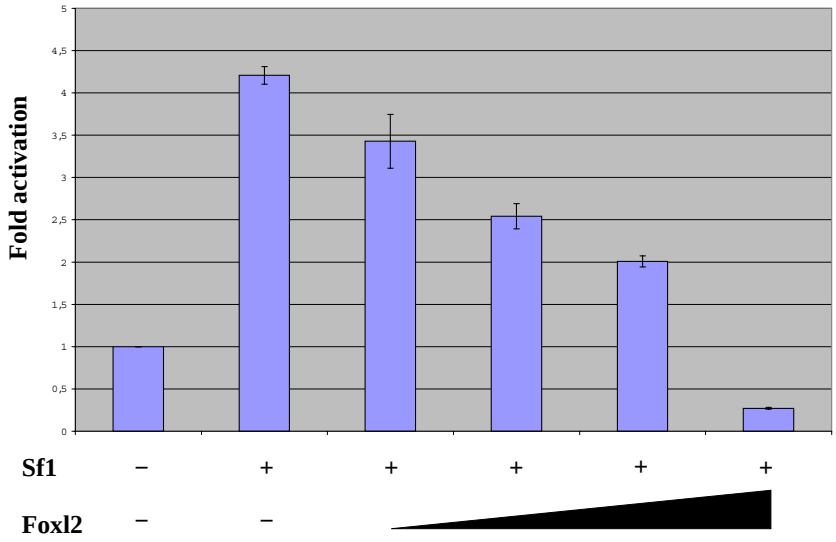
Experiments in tilapia suggest that FOXL2 and SF1 can physically interact (Wang et al., 2007). A similar interaction could be occurring in the developing mouse gonad, resulting in a repression of SF1-mediated transcriptional activation of TESCO. To test this hypothesis, a synthetic sequence element containing only SF1 binding sites and no FOX sites, was cloned into the luciferase vector (Figure 4.8a). This luciferase reporter construct was indeed activated in co-transfection assays by SF1 and addition of FOXL2 resulted in a dosage dependent repression (Figure 4.8a). This result was similar to the FOXL2-mediated repression of TESCO (Figure 3.4b). As no forkhead binding sites are present in this syn-

thetic element, the repression cannot be due to FOXL2 binding to FOX sites but must be mediated through a different mechanism, e.g. direct interaction between FOXL2 and SF1.

To analyse this hypothesis, a GST-FOXL2 fusion protein and a ^{35}S-labelled SF1 protein were generated. Co-immunoprecipitation assays with the two proteins were performed, showing that FOXL2 and SF1 can indeed directly interact (Figure 4.9). No interaction could be detected in the controls with ^{35}S-SF1 on its own or ^{35}S-SF1 together with the GST control.

Next, the effect of FOXL2 on the DNA-binding ability of SF1 was analysed. The synthetic SF1-binding sequence, which contained SF1 but no FOX binding sites, was radioactively labelled. This probe was then incubated with SF1 and increasing amounts of FOXL2 (Figure 4.10). As expected, binding of SF1 to the DNA was clearly visible. Interestingly, a weak interaction could also be detected in the sample where only FOXL2 (and not SF1) was added to the DNA, indicating that FOXL2 might be able to bind to DNA containing SF1 binding sites. Increasing amounts of FOXL2 resulted in an attenuation of the band representing SF1 bound to DNA. This suggests that FOXL2 competes with SF1 for binding to SF1 binding sites. As the DNA contains more than one SF1 binding site, it is possible that several SF1 and FOXL2 proteins could simultaneously bind to the sequence. The formation of such multimers could result in the an even higher bandshift and possibly explain the upper bands seen in this assay.

(a)



(b)

Figure 4.8: **Effect of FOXL2 on SF1-mediated activation.**
A synthetic sequence containing only SF1 binding sites was cloned into the Luciferase vector (a). In co-transfection assays, this construct was activated by SF1. Addition of increasing amounts of FOXL2 resulted in a repression in a dosage-dependent manner (b). Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

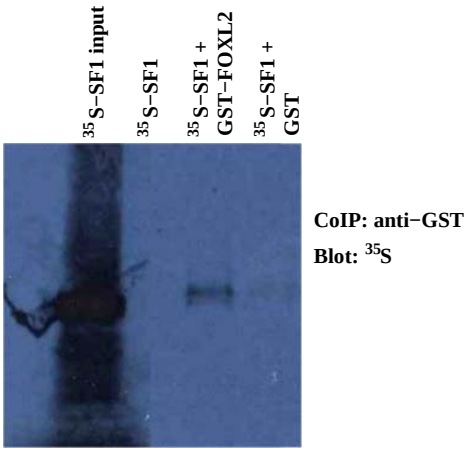


Figure 4.9: **Direct physical interaction between FOXL2 and SF1.**
Co-Immunoprecipitation of GST-FOXL2 and ^{35}S-SF1 showing an interaction between the two proteins.

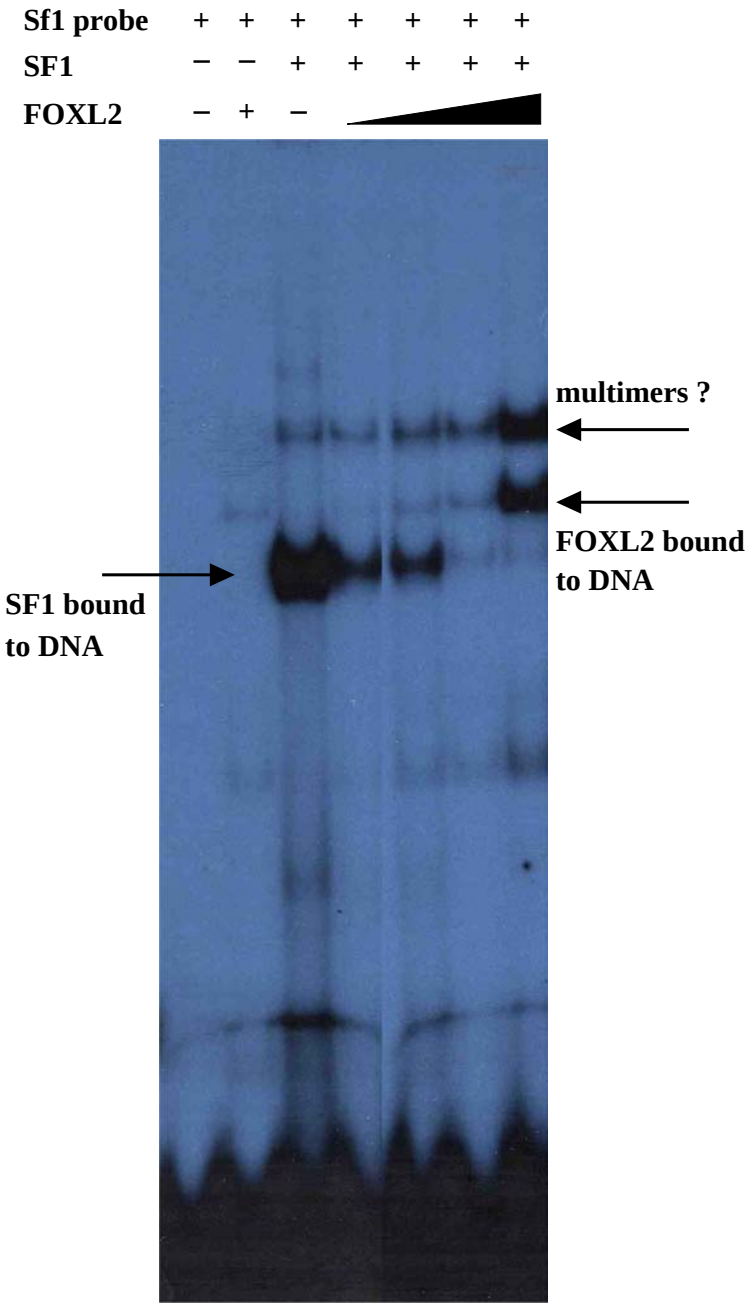


Figure 4.10: **FOXL2 interferes with SF1 binding to DNA.**
A radioactively labelled probe containing only SF1 binding sites was incubated with SF1 or FOXL2 or both proteins. SF1 was able to bind to its binding sites as indicated by the band shift. When FOXL2 was added on its own a weak band appears indicating the possibility of FOXL2 binding to the SF1 site. Increasing amounts of FOXL2 resulted in a decrease of SF1 bound to DNA and an increase of FOXL2 bound to DNA, suggesting a competition for binding between the two proteins.

4.3 Conclusions

It has been shown so far that FOXL2 represses TESCO activity *in vitro*. In this chapter, it was assessed how FOXL2 mediates this repression effect. As FOXL2 is a transcription factor which can activate or repress its target genes by binding to its consensus DNA binding sites, the analysis first asked whether FOXL2 represses TESCO activity by binding to possible binding sites in the TESCO sequence. Mutation of the ten putative forkhead factor binding sites in the TESCO sequence did not abolish the repression mediated by FOXL2 in co-transfection assays. However, it is possible that the TESCO element contains additional forkhead factor binding sites which differ from the consensus forkhead binding site. It is known that some groups of forkhead factors indeed bind to sequences which only partially match the consensus site (Carlsson and Mahlapuu, 2002). Thus, FOXL2 could still be repressing TESCO activity via potential non-consensus binding sites in the *in vitro* assays.

FOXL2 and ERα were found to physically interact with each other in co-immunoprecipitation assays (Uhlenhaut et al., 2009; Kim et al., 2009). While the combined mutation of all FOX sites and EREs found in the TESCO element still resulted in repression of TESCO activity by FOXL2, it abolished the synergistic repression effect of ERα together with FOXL2 *in vitro*. Similarly, simultaneous mutation of all FOX and ERE sites in the TESCO:CFP reporter construct, resulted in in a de-repression of TESCO activity in adult ovaries *in vivo* (Uhlenhaut et al., 2009). These results indicate an involvement of both factors in the repression of TESCO and the importance of forkhead factors for estrogen

receptor function. However, the exact mechanism of how FOXL2 and ERα repress TESCO remains to be investigated. Furthermore, it is possible that the mutated TESCO sequence still contains some minor interaction sites for FOXL2, which do not play an important role in the normal physiological environment *in vivo*. However, due to the artificial system of the co-transfection assays *in vitro*, the excessive amount of FOXL2 available might still repress the TESCO element via these sites. Thus, the repression of the mutated TESCO element *in vitro* in contrast to the de-repression seen *in vivo* might be due to a dosage effect of FOXL2.

Interestingly, deletion of different parts of the TESCO element resulted in the same level of repression by FOXL2 as the wild-type TESCO sequence. These results suggest that FOXL2 could be additionally repressing TESCO activation via a different mechanism. Co-immunoprecipitation analysis revealed that FOXL2 and SF1 are able to physically interact with each other. Thus, it is possible that FOXL2 could mediate its repression effect via SF1, by repressing the SF1-mediated transcriptional activation of TESCO. Recently, a paper was published which identified a new FOXL2-specific DNA binding site GTCAAGG(T/C)CA (Benayoun et al., 2008) which shows close similarity to the SF1-binding site (T/C)(T/C)AAGG(T/C)C(G/A). This suggests the possibility that FOXL2 might be able to bind to the SF1 DNA site. Indeed, gel mobility shift assays showed that FOXL2 can bind to a DNA sequence containing only SF1-binding sites and no FOX sites. Moreover, increasing amounts of FOXL2 resulted in a decrease of SF1 bound to its DNA binding site. Likewise, increasing amounts of FOXL2 led to a dosage-dependent repression of an luciferase reporter construct contain-

ing the same DNA sequence in co-transfection assays. These results suggest a mechanism involving competition between FOXL2 and SF1 for binding to the SF1 site. In this hypothesis, binding of SF1 could result in TESCO activation whereas binding of FOXL2 might result in TESCO repression.

In summary, these results suggest a cooperation of FOXL2 and ERα in repressing TESCO activity. It is possible that the two proteins interact as a functional unit which can either bind to FOX sites or EREs to repress the TESCO element. However, the data obtained here also suggest the possibility of a second mechanism of FOXL2-mediated repression involving SF1. There could be several hypotheses: (i) FOXL2 might physically interact with SF1 and thereby reduce the amount of SF1 available to activate TESCO, (ii) FOXL2 might interact with SF1 which is bound to its DNA binding site in the TESCO element, thereby inhibiting its activation capability and (iii) FOXL2 might be competing with SF1 for binding at the TESCO element, resulting in a repression of TESCO rather than is activation.

The results obtained in this thesis so far have provided evidence that FOXL2 can repress TESCO activity *in vitro* and might therefore be involved in the regulation of *Sox9* in the XX gonad *in vivo*. To investigate this further, the repressing effect of FOXL2 on TESCO activity was analysed next *in vivo*.

Chapter 5

The effect of *Foxl2* on TESCO activity *in vivo*

5.1 Introduction

The female-specific expression pattern of *Foxl2* in the gonad is highly conserved amongst vertebrates, and also in species with very distinct mechanisms of sex determination. For example, in the red-eared slider turtle (*Trachemys scripta*), in which the sex of the offspring is dependant on the egg incubation temperature, the *Foxl2* transcript levels are higher at female promoting temperatures than at male promoting temperatures (Loffler et al., 2003). In the tilapia fish (*Oreochromis niloticus*), *Foxl2* is highly expressed in XX individuals at the critical period of sex determination (between 5 to 6 days after hatching) whereas it is barely detectable in XY individuals (Ijiri et al., 2008). In rainbow trout (*Oncorhynchus mykiss*), XY fish treated with estrogens up-regulate *Foxl2* expression and become feminised, whereas XX fish treated with androgens show signs of masculinisation and down-regulate *Foxl2* expression (Baron et al., 2004). In frog

(*Xenopus laevis*), *Foxl2* expression is higher in the female ZW gonad than in the male ZZ gonad (Okada et al., 2009). A similar sexual dimorphic pattern is also observed in the chick, where *Foxl2* is specifically expressed in the ZW gonad from the beginning of sex determination (Loffler et al., 2003). In the mouse, *Foxl2* is expressed in the somatic cells of the XX gonad from 12.5 dpc onwards, whereas it is not detected in XY gonads (Uda et al., 2004). The conserved female-specific expression pattern of *Foxl2* in the developing gonad suggests the potential of this gene to act as a conserved factor in ovarian development. However, the molecular mechanisms explaining how the transcription factor FOXL2 regulates ovarian development and differentiation are not yet fully understood.

Mice heterozygous for a null mutation of *Foxl2* display no phenotype, whereas homozygotes mutant mice ($Foxl2^{-/-}$) are born with open necrotising eyes, eyelid hypoplasia, craniofacial defects and smaller body size, which are features comparable to human BPES (Uda et al., 2004). In addition, XX $Foxl2^{-/-}$ mice show hypoplastic uterine tubes and are infertile, whilst XY $Foxl2^{-/-}$ mice remain fully fertile and testis development is unaffected. The same phenotype was found in mice carrying a *LacZ* gene insertion into the *Foxl2* locus ($Foxl2^{LacZ}$) resulting in a null allele (Schmidt et al., 2004). Directly after birth the numbers of primordial follicles and oocytes are similar between wild-type and $Foxl2^{-/-}$ mutant ovaries, but 2 weeks postnatally, when secondary follicles are normally surrounded by two cuboidal granulosa cell layers in the wild-type ovary, the mutant follicles contain only one layer of granulosa cells. Moreover, these cells have not yet completed the transition from squamous to cuboidal type. At 8 weeks, mutant ovaries are significantly smaller than wild-type and mutant follicles contain significantly fewer

granulosa cells due to decreased proliferation. In addition, no quiescent primordial follicles are found indicating a depletion of the primordial follicle pool (Uda et al., 2004). The expression of *Sox9* and some other markers of testis differentiation such as *Fgf9*, *Fgfr2*, *Dhh*, *Wt1*, *Sf1*, *Gata4* is up-regulated in the mutant ovary 1 week after birth (Ottolenghi et al., 2005).

Recently, our collaborators Henriette Uhlenhaut and Mathias Treier, have generated a mouse line carrying a conditional *Foxl2* allele ($Foxl2^{flox/flox}$). To determine *Foxl2* function in the adult ovary, an ubiquitously expressed inducible Cre recombinase allele (Rosa26:CreERT2) was bred into this line to allow the deletion of *Foxl2* upon administration of tamoxifen (thus giving $Foxl2^{\Delta/\Delta}$). Analysis 3 weeks after the tamoxifen administration to 8 week old mice, revealed XX gonadal sex reversal (Uhlenhaut et al., 2009). The gonads of these mice contained structures resembling seminiferous tubules with Sertoli-like cells as indicated by tripartite nucleoli and veil-like cytoplasmic extensions pointing towards the lumen. The tubule-like structures were also surrounded by a prominent basal lamina. One week after tamoxifen administration, the morphology of the follicles and the oocytes in $Foxl2^{\Delta/\Delta}$ gonads were still intact, but the granulosa cells surrounding the oocytes were expressing *Sox9*. These cells started to transdifferentiate into Sertoli-like cells despite the presence of an oocyte, suggesting that granulosa cells undergo cell autonomous reprogramming into Sertoli-like cells upon loss of *Foxl2*. A number of other Sertoli cell markers were also up-regulated in these cells, including *Gata1*, *Tif2*, *Dmrt1* and *Dhh*. Leydig cell markers, such as *Hsd17b3* (the rate-limiting enzyme in testosterone production in the testis) were also up-regulated in the XX $Foxl2^{\Delta/\Delta}$ gonads. Moreover, *Hsd17b3* was found

to be expressed in a similar pattern to adult testis, suggesting the presence of Leydig-like cells. Furthermore, similar levels of testosterone were detected in the blood of XX *Foxl2*$^{\Delta/\Delta}$ mice compared with XY wild-type littermates. Looking at events immediately after tamoxifen administration revealed that FOXL2 protein was still present 2 days after whereas SOX9 was not yet expressed. However, SOX9 was detected already at high levels 4 days after tamoxifen administration, by which time FOXL2 expression had disappeared. No evidence could be found of any overlap, suggesting that expression of FOXL2 and SOX9 are mutually exclusive.

All the evidence suggested that FOXL2 is required in granulosa cells of the post-natal ovary to antagonise *Sox9* expression and actively prevent Sertoli cell differentiation and subsequent masculinisation of the gonad. The data presented in the previous chapters of this thesis showed that FOXL2 can repress *Sox9* expression *in vitro* via the TESCO enhancer element (see Chapter 3).

To test whether the same mechanism occurs *in vivo* *Foxl2*^{LacZ} mice, which were generated by the replacement of most of the *Foxl2* coding region by β-galactosidase (Schmidt et al., 2004), were crossed to a transgenic mouse line expressing a CFP reporter under the control of the TESCO enhancer element (TESCO:CFP) (Sekido and Lovell-Badge, 2008). The expression of CFP in the TESCO:CFP line correlates with the expression of endogenous SOX9: in XY gonads CFP is expressed in Sertoli cells from 11.5 dpc until adult stages, whereas in XX gonads CFP is not expressed at all in granulosa cells (Figure 5.1). If FOXL2 represses *Sox9* expression via the TESCO element *in vivo*, loss of *Foxl2* in the XX go-

nad should lead to a de-repression of TESCO and therefore activation of CFP expression in the XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ ovary.

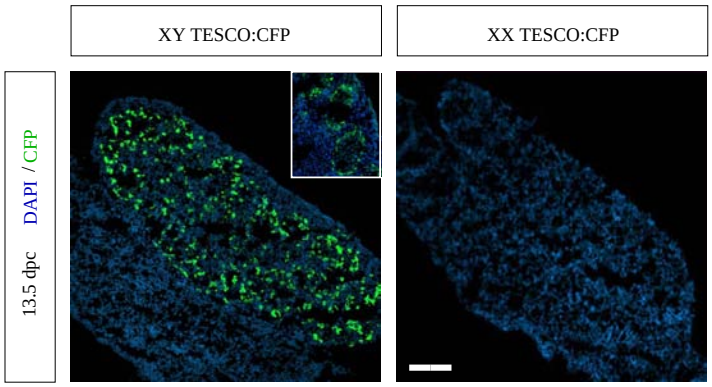


Figure 5.1: **CFP expression in the TESCO:CFP reporter line *in vivo*.** Immunohistochemistry showing CFP expression in XY, but not XX gonads, of the TESCO:CFP reporter line at 13.5 dpc, equates to the expression pattern of *Sox9*. Scale bar = 100μm; insert shows 2.5x higher magnification.

5.2 Results

5.2.1 De-repression of TESCO in *Foxl2* mutant mice

In the original paper describing the *Foxl2*$^{LacZ/LacZ}$ phenotype, it was reported that homozygotes are born at the expected Mendelian ratio, although about 95% of the mutant animals die before weaning (Schmidt et al., 2004). However, after being bred onto a C57BL/6 background for several generations, it was not possible to find any homozygous mutant mice amongst the litters born or even amongst the embryos harvested at 12.5 dpc (a total of 207 mice and 114 embryos were generated from heterozygous *Foxl2* matings on the C57BL/6 background). As the original mutation was maintained on a mixed background (129/BlackSwiss/CD1), this result suggests a variable penetrance of the *Foxl2* mutation in different mouse strains and a high sensitivity of the C57BL/6 background for the *Foxl2* mutation. To circumvent this problem, the *Foxl2* mutant mice were first bred onto an MF1 hybrid background, which resulted in the appearance of live born mutant mice after four generations. These *Foxl2*[MF1] homozygous mutant mice were smaller than wild-type and heterozygous littermates and they were born with open eyes and craniofacial defects as described in Schmidt et al. (2004). The *Foxl2*[MF1] mice were then bred with TESCO:CFP reporter mice to generate the desired *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ genotype.

To determine the possible de-repression of TESCO activity, XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads were collected and compared to control gonads from XY *TESCO:CFP; Foxl2*$^{LacZ/wt}$ and XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ mice at different time points during embryogenesis (12.5 dpc to 18.5 dpc) and after birth (P0 to

8 weeks). The gonads were then sectioned and analysed for CFP expression. Ovaries of XX heterozygous and homozygous *Foxl2* mutant mice are indistinguishable directly after birth (P0). However, from 7 dpp onwards morphological differences could be found between the two genotypes and the ovarian dysgenic phenotype of XX homozygous mutants became more prominent with age. From 4 weeks onwards, it was clearly evident that oocytes (asterisks) in *Foxl2*$^{LacZ/LacZ}$ ovaries are surrounded by fewer layers of granulosa cells (arrows), compared to several layers of granulosa cells in the *Foxl2*$^{LacZ/wt}$ ovaries (Figure 5.2), as described in the original paper (Schmidt et al., 2004).

As expected, during embryonic development, CFP was expressed in the Sertoli cells within the testis cords in XY *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads, whereas it was absent in XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads between 12.5 dpc and 18.5 dpc. No CFP expression could be detected in the gonads of XX TESCO:CFP; Foxl2$^{LacZ/LacZ}$ mice at any stage during embryogenesis. However, just after birth (P0) a very few CFP-positive cells were found (Figure 5.3).

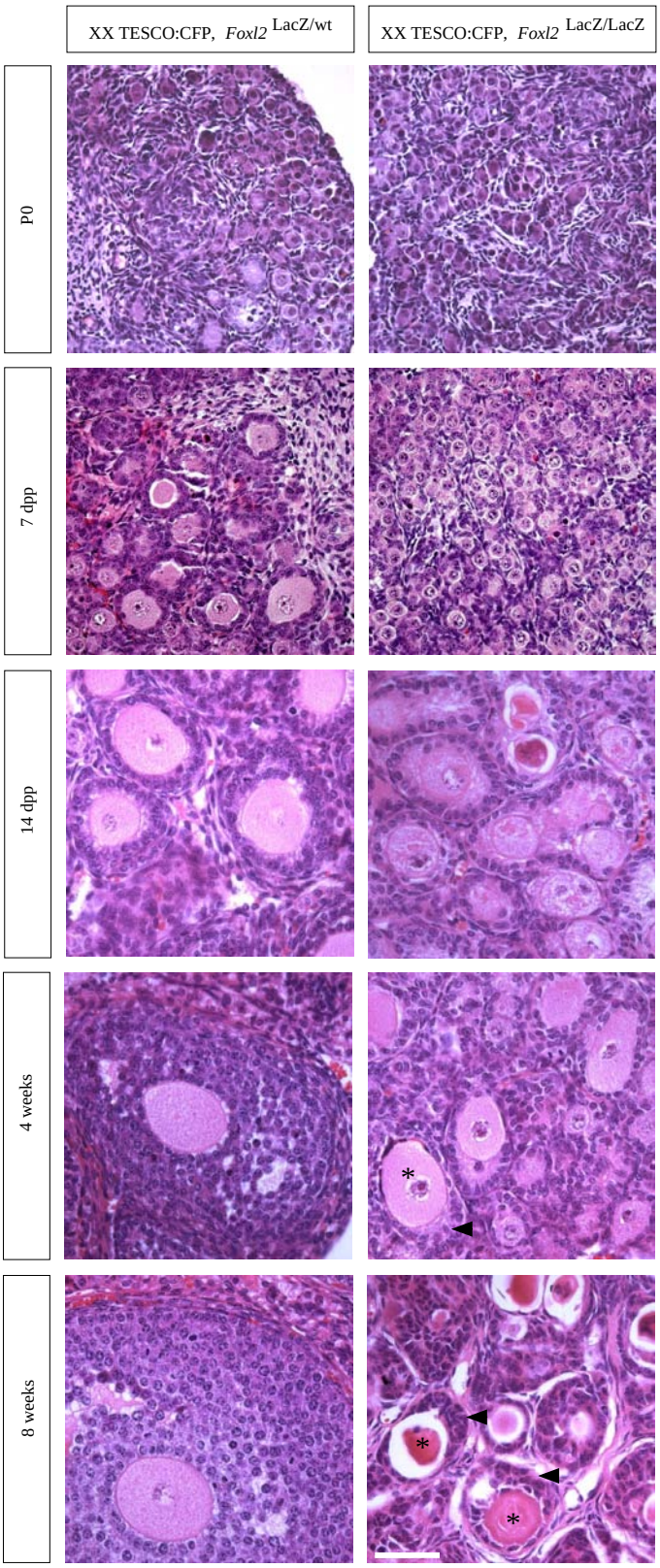


Figure 5.2: **Morphology of XX *Foxl2*$^{LacZ/LacZ}$ gonads.**
H+E staining of XX *Foxl2*$^{LacZ/wt}$ and *Foxl2*$^{LacZ/LacZ}$ gonads at different time points after birth and during adulthood. No morphological difference could be seen at P0. From 4 weeks of age it was evident that fewer layers of granulosa cells (arrows) surround the oocytes (asterisks) in *Foxl2*$^{LacZ/LacZ}$ ovaries. Scale bar = 50μm.

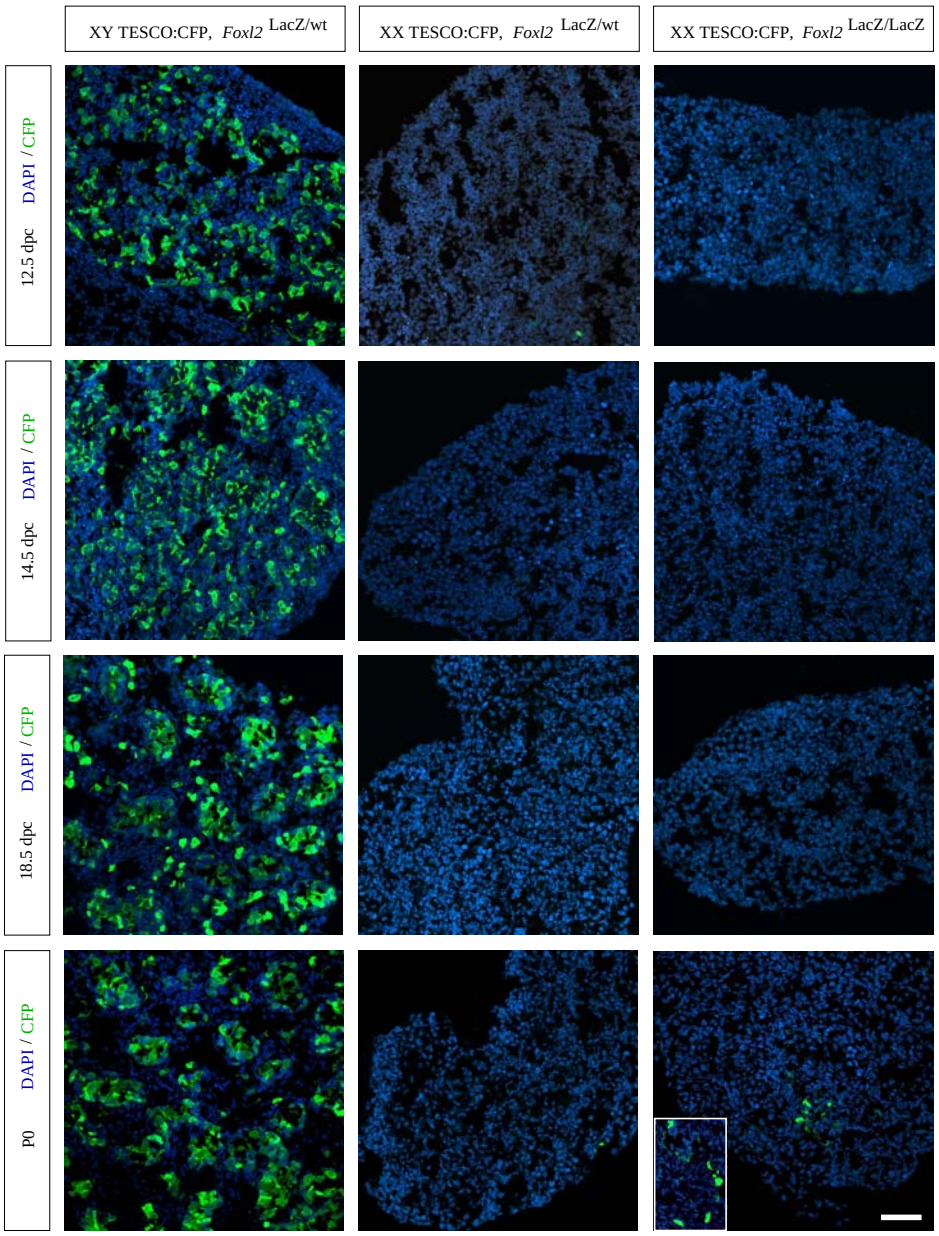


Figure 5.3: **TESCO activity in *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads during embryogenesis.**
Cryosections of XY and XX gonads of TESCO:CFP mice, with heterozygous and homozygous loss of *Foxl2*, at different time points during embryogenesis and just after birth (P0). Immunostaining shows DAPI in blue and CFP in green. TESCO:CFP expression could be detected in Sertoli cells in the XY gonads (left panel), whereas it was absent in XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads (middle panel). No de-repression of TESCO activity was found in XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads during embryogenesis, but a few CFP-positive cells could be detected just after birth (right panel). Scale bar = 50μm; insert shows 2.5x higher magnification of CFP-positive cells in ovary with homozygous loss of *Foxl2*.

At postnatal stages, CFP expression was detected in the Sertoli cells of all XY *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads from 7 dpp until 8 weeks of age. During this time frame the normal reorganisation of the testis cords could be observed, including the migration of Sertoli cells towards the centre of the cords just after birth, the migration of the Sertoli cell nuclei back to the basal area of the seminiferous tubules after puberty and the finger-like cytoplasmic extension of adult Sertoli cells from the basal to the adluminal compartment of the seminiferous tubules (Figure 5.4). In XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads, no CFP expression was seen before 4 weeks of age, except for a non-specific staining in oocytes. However, at 4 and 8 weeks of age, CFP expression was detected in some theca cells surrounding the follicles. It has been reported that *Sox9* is expressed in the inner layer of theca cells in the adult ovary but does not coincide with the expression of the steroidogenic markers P450 and 3βHSD, suggesting that the SOX9-positive cells are a subpopulation of non-steroidogenic theca cells (Notarnicola et al., 2006). In the XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ mice, CFP expression was detected in all gonads from 7 dpp to 8 weeks of age (Figure 5.4). The CFP-positive cells in the homozygous *Foxl2* mutant gonads were mostly found in the remaining layer surrounding the oocytes.

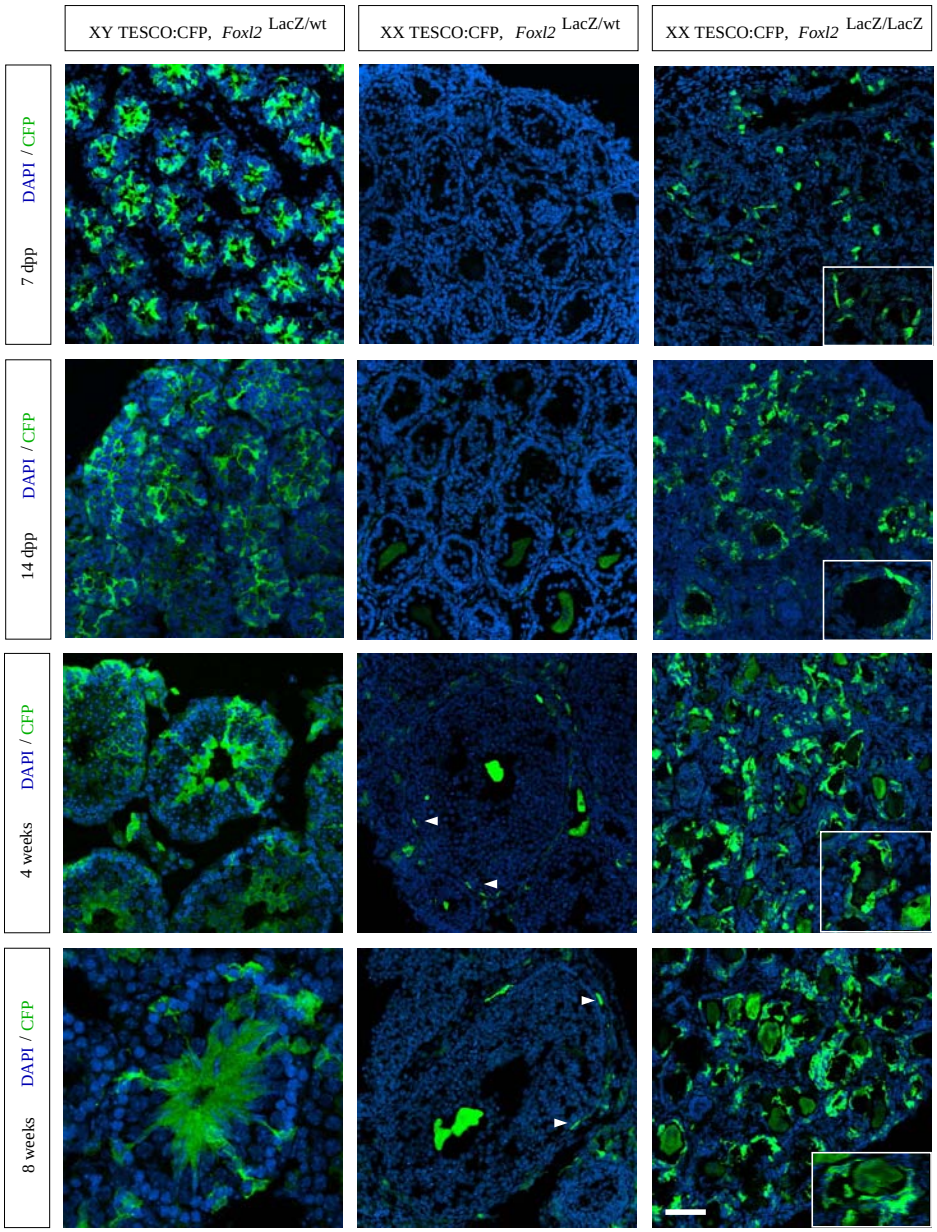


Figure 5.4: **TESCO activity in postnatal *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads.**
Cryosections of XY and XX gonads of TESCO:CFP mice, with heterozygous and homozygous loss of *Foxl2*, at different time points postnatally. Immunostaining shows DAPI in blue and CFP in green. CFP expression was detected in Sertoli cells in XY gonads (left panel) and in a potential subpopulation of theca cells in XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads from 4 weeks onwards (white arrows, middle panel). De-repression of TESCO could be seen in XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads in cells surrounding the oocytes at all stages analysed (right panel). Scale bar = 50μm; inserts show 2.5x higher magnifications.

5.2.2 De-repression of SOX9 in *Foxl2* mutant mice

To determine whether de-repression of TESCO activity coincided with de-repression of endogenous *Sox9*, the expression of *Sox9* was analysed in *Foxl2* mutant gonads during embryonic development and after birth.

While *Sox9* was expressed normally in the Sertoli cells of embryonic XY gonads, no *Sox9* expression could be detected in either XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ or XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads between 12.5 dpc and 18.5 dpc (data not shown). After birth, *Sox9* expression was found in the Sertoli cells of XY gonads from P0 to 8 weeks of age and in a few cells in the ovaries of 4 and 8 week old XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ mice, which might represent the sub-population of theca cells as noted above (Notarnicola et al., 2006). However, endogenous SOX9 was detected in the ovaries of XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ mice from 7 dpp to 8 weeks of age, in most of the residual cells surrounding the oocytes (Figure 5.5). Co-staining for nuclear SOX9 and cytoplasmic CFP revealed the expression of both proteins in the same cells. Interestingly, whereas CFP expression was found in ovaries of XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ mice from P0 (Figure 5.3), SOX9 was not detected until later (Figure 5.5).

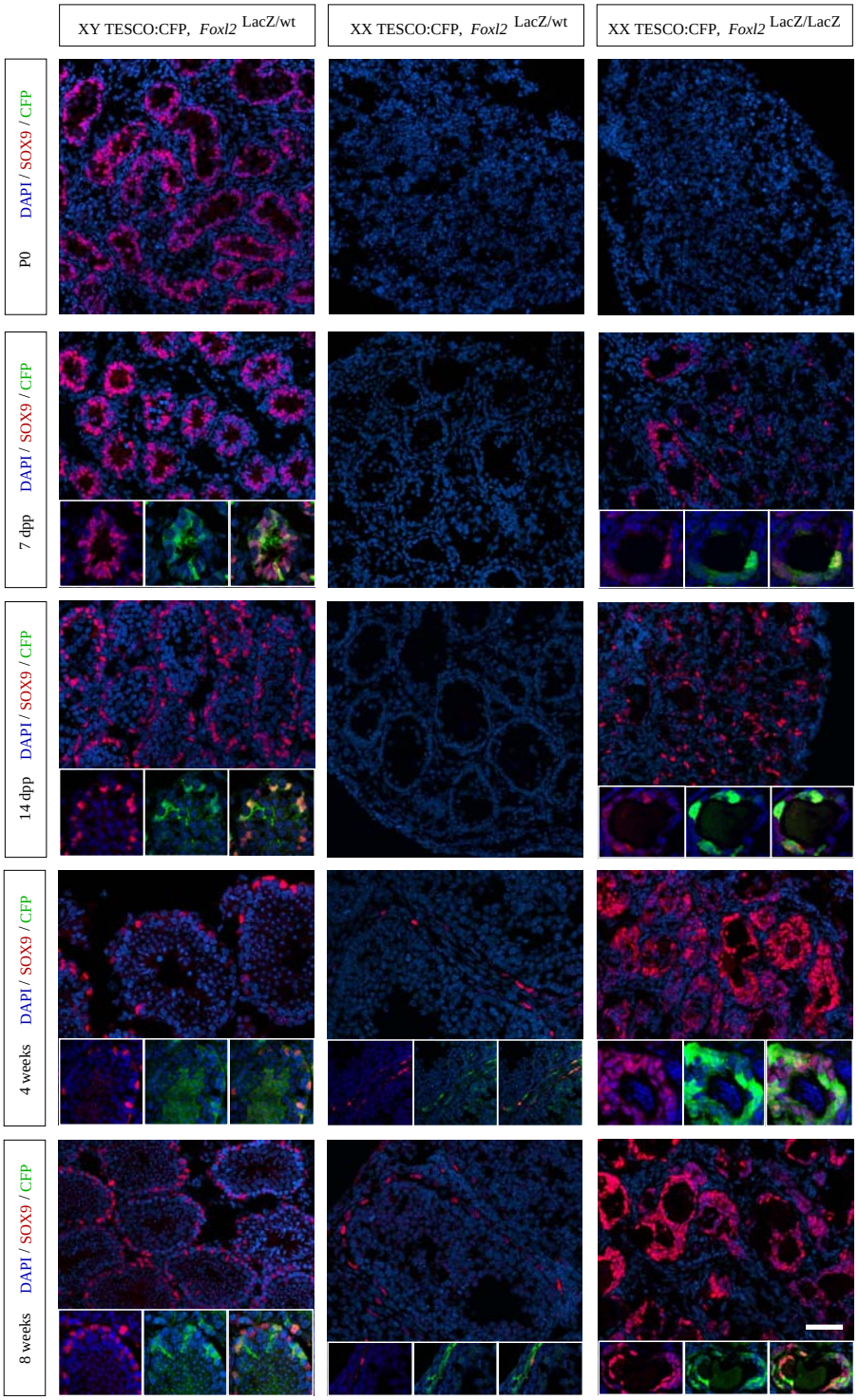


Figure 5.5: **Endogenous *Sox9* expression in postnatal *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$.**
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of *Foxl2* at different time points postnatally. Immunostaining shows DAPI in blue, CFP in green and SOX9 in red. Inserts show higher magnifications. CFP and SOX9 co-localised in Sertoli cells in the XY gonads (left panel), in a possible subpopulation of theca cells in the XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads (middle panel) and in cells surrounding the oocytes in XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads (right panel). Scale bar = 50μm; inserts show 2.5x higher magnifications.

As mentioned above, expression from both TESCO and *Sox9* could be seen in the cells which surround the oocytes in a typical granulosa cell manner. To determine whether indeed the remaining granulosa-like cells are the cells up-regulating *Sox9*, the *Foxl2* mutant gonads were analysed for the expression of *LacZ*, a reporter gene inserted into the *Foxl2* locus and therefore expressed under the control of the endogenous *Foxl2* promoter. In double immunostainings, the gonadal expression of SOX9 and β-galactosidase was analysed in 4 week old mice (Figure 5.6). XY *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads showed *Sox9* expression in the Sertoli cells, but no expression of β-galactosidase. On the other hand, in XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads, β-galactosidase could be found predominantly in the granulosa cells, but also in some interstitial cells (large white arrow). Although it has been shown that *Foxl2* is also expressed in a subpopulation of theca cells (Uhlenhaut et al., 2009), in this analysis no β-galactosidase activity could be detected in any theca cells. As described before, *Sox9* expression could be detected in some cells of the XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads that might represent a subpopulation of theca cells (small white arrow). In the XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads, the cells surrounding the oocytes expressed both *Sox9* and β-galactosidase. However, some cells in the XX homozygous mutant gonad were positive for β-galactosidase but not for SOX9 and might correspond to the interstitial cells which were also found in the XX *Foxl2* heterozygous mutant gonads.

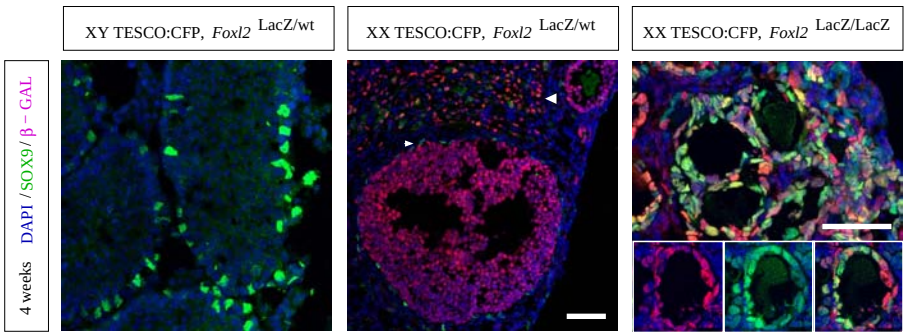


Figure 5.6: **Co-localisation of SOX9 and β-galactosidase.**
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of *Foxl2* at 4 weeks of age. Immunostaining shows DAPI in blue, SOX9 in green and β-galactosidase in red. In XY gonads, SOX9 but not β-galactosidase was expressed. In XX *TESCO:CFP; Foxl2*$^{LacZ/wt}$ gonads, SOX9 was expressed in cells likely to represent a subpopulation of theca cells (small white arrow) and β-galactosidase was expressed in granulosa cells and some interstitial cells (large white arrow). In XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads, SOX9 and β-galactosidase co-localised in the cells surrounding the oocytes. Scale bar = 50μm; inserts show 2.5x higher magnifications.

5.2.3 The impact of oocyte depletion on the supporting cell lineage

It has been suggested that oocytes are required for the maintenance of granulosa cells and that the ovarian pathway is actually under the control of the germ cells (Burgoyne, 1988; McLaren, 1991). If these cells are lost at the time of folliculogenesis, testis-like structures form in cultured fetal rat ovaries (Prépin and Hida, 1989). The same effect can be seen naturally in freemartin cattle, where female embryos have a vascular connection to their male twins. Here, the oocytes gradually degenerate and testicular cords form in the ovary, including Sertoli cells which produce AMH and Leydig cells which produce testosterone (McLaren, 1991). Other studies have shown that oocytes can interfere with the organisation of testis cords and thus might be involved in antagonising testis

differentiation (Yao et al., 2003). Based on this evidence it has been proposed that the loss of oocytes could by itself cause the transdifferentiation of granulosa to Sertoli-like cells.

To investigate this possibility, oocytes were specifically depleted in neonatal mice and *Sox9* expression was analysed to determine whether the loss of oocytes could result in a de-repression of *Sox9*. For this, mice carrying a conditionally expressed gene for diphtheria toxin fragment A (DTA) in the ROSA26 locus were used (R26DTA). These express the active DTA only upon Cre-mediated recombination (Ivanova et al., 2005). Diphtheria toxin is secreted by the parasite *Corynebacterium diphtheriae* and consists of subunit A and B. Subunit B mediates the internalisation of the toxin by binding to its receptor, whereas subunit A terminates protein synthesis and eventually leads to apoptosis of the cell (Maxwell et al., 1986). The R26DTA mice were bred to mice carrying Cre-recombinase under the control of the germ-line specific promoter of the zona pellucida 3 gene (Zp3:Cre), which is specifically expressed in oocytes (De Vries et al., 2000). Zp3:Cre is active in ovaries from 5 dpp onwards, in primary, secondary, and later follicular stages, but not in adjacent somatic cells (Lan et al., 2004). Therefore, upon Zp3:Cre mediated recombination, the DTA will be active exclusively in oocytes from the primary follicular stage onwards, resulting in a global loss of oocytes.

To investigate the possible up-regulation of *Sox9* expression upon oocyte loss, gonads of XY *Zp3:Cre; R26DTA*, XX *R26DTA* and XX *Zp3:Cre; R26DTA* mice were collected at different time points after birth (P0 to 8 weeks). The gonads were then sectioned and analysed for *Sox9* expression. As expected, *Sox9* expres-

sion could be detected in Sertoli cells of XY *Zp3:Cre; R26DTA* gonads at all ages between P0 and 8 weeks of age and in the putative subpopulation of theca cells of four and eight week old XX Zp3:Cre-negative *R26DTA* gonads (white arrows). As Zp3:Cre is not active at P0, ovaries of XX *Zp3:Cre; R26DTA* mice just after birth were similar to ovaries of XX *R26DTA* mice, indicating that indeed the DTA had no effect in those ovaries yet. The first morphological differences could be found at 7 dpp and became more evident with age, resulting eventually in a complete oocyte-depleted ovary at 4 and at 8 weeks of age. Adult XX *Zp3:Cre; R26DTA* ovaries were drastically smaller compared to those of XX Zp3:Cre-negative littermates. Despite the severely altered morphology, up-regulation of *Sox9* expression was not detected at any stage between P0 and 8 weeks of age in these XX *Zp3:Cre; R26DTA* ovaries (Figure 5.7).

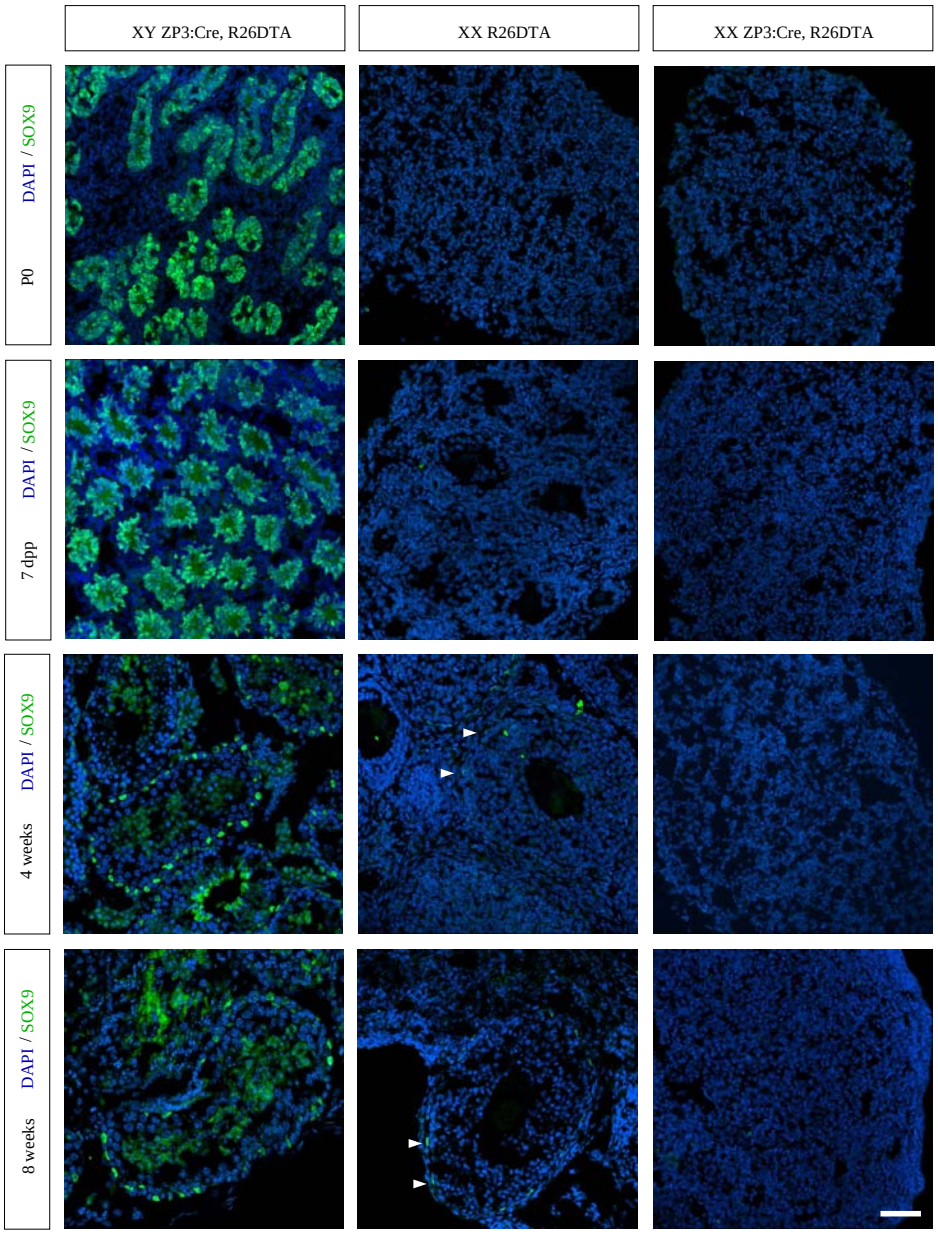


Figure 5.7: ***Sox9* expression in gonads of *Zp3:Cre; R26DTA* mice.** Cryosections of XY and XX gonads of R26DTA mice with or without Zp3:Cre at different time points postnatally. Immunostaining shows DAPI in blue and SOX9 in green. SOX9 could be detected in Sertoli cells of XY *Zp3:Cre; R26DTA* gonads (left panel) and in a few cells in XX *R26DTA* gonads (white arrows, middle panel). SOX9 was not detected in XX *Zp3:Cre; R26DTA* gonads (right panel). Scale bar = 50μm.

5.3 Conclusions

In this chapter the effect of *Foxl2* on TESCO activity was analysed *in vivo* using *Foxl2*^{LacZ} mice crossed to TESCO:CFP reporter mice. Homozygous loss of *Foxl2* did not have any effect on either TESCO activity or endogenous *Sox9* expression during embryogenesis. However, after birth homozygous loss of *Foxl2* in XX gonads resulted in the de-repression of TESCO activity and thus activation of CFP expression from P0 onwards. Just after birth, very few CFP-positive cells could be detected in the gonads of these XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ mice and their number increased with age. From 4 weeks onwards, it was clear that the cells which show de-repression of TESCO were mainly the cells surrounding the oocytes. In addition, endogenous *Sox9* expression was detected in ovaries of *Foxl2* homozygous, but not heterozygous, mutant mice. *Sox9* expression coincided with CFP expression in the cells surrounding the oocytes. These cells also expressed β-galactosidase, which is largely specific to granulosa cells as it is expressed under the endogenous *Foxl2* promoter. All this evidence indicates that indeed the remaining supporting cells which are surrounding the oocytes in the XX Foxl2$^{LacZ/LacZ}$ gonads up-regulate *Sox9* expression upon loss of *Foxl2* and thus switch their cell fate from the female supporting lineage to male Sertoli-like cells.

Interestingly, TESCO and endogenous *Sox9* became de-repressed at different time points in the XX Foxl2$^{LacZ/LacZ}$ gonads. While a few CFP-positive cells could already be detected at P0, *Sox9* was still repressed. There are several possible explanations for these results: (i) endogenous *Sox9* expression might be repressed

via different mechanisms compared to the TESCO element in XX gonads at the time of birth, (ii) the fact that only very few CFP-positive cell could be found at this time point suggests that other genes might be involved in the repression of TESCO activity (and therefore *Sox9* expression) around birth, (iii) TESCO:CFP is a transgene and might be inserted at a more accessible position in the genome than the endogenous *Sox9* gene. Thus, it is possible that the differential de-repression profile of TESCO:CFP transgene and endogenous *Sox9* might be due to epigenetic differences.

Both TESCO and *Sox9* became clearly de-repressed with increasing age in the XX Foxl2$^{LacZ/LacZ}$ gonads, indicating that *Foxl2* might be the important gene in repressing *Sox9* in the adult. These results match with the findings of our collaborators in adult XX *Foxl2*$^{\Delta/\Delta}$ mice, where endogenous *Sox9* expression could be detected after the complete loss of FOXL2. Moreover, when bred on the TESCO:CFP background, CFP expression was also up-regulated in a typical granulosa cell location of the adult ovary after *Foxl2* deletion (Uhlenhaut et al., 2009).

For a long time it had been proposed that oocytes are essential to maintain granulosa cell fate. In different models the degeneration of oocytes was associated with the appearance of testis cord like structure in XX gonads and the transdifferentiation of granulosa cells into Sertoli-like cells (McLaren, 1991). When 13.5 dpc XX gonads were grafted to the kidney capsule of adult mice, they developed cord like structures and started to express *Sox9* (Morais da Silva et al., 1996). However, the signals responsible for this testis development of the grafted XX

gonads are not known. To determine whether the loss of oocytes could result in a de-repression of *Sox9*, oocytes were specifically depleted by activating the DTA upon Zp3:Cre mediated recombination. In the *Zp3:Cre; R26DTA* mice, the active DTA caused apoptosis in oocytes from the primary follicular stage, resulting in a much smaller, completely oocyte-depleted ovaries from 7 dpp onwards. Although follicular growth was significantly impaired, no *Sox9* expression was detected in these ovaries, indicating that the supporting cells in these oocyte-depleted ovaries did not switch to Sertoli-like cells. These results are consistent with the data of our collaborators, who generated *Gdf9:Cre; R26DTA* mice to analyse oocyte depletion in the ovary. *Gdf9* is expressed in oocytes from the primordial follicle stage onwards at 3 dpp, whereas *ZP3* expression starts at 5 dpp (Lan et al., 2004). Analyses of these *Gdf9:Cre; R26DTA* mice showed similar results: no *Sox9* expression could be detected in the ovaries upon oocyte loss (Uhlenhaut et al., 2009). These results differ from the ones obtained in the analyses of the $Foxl2^{LacZ/LacZ}$ mice which showed a postnatal de-repression of *Sox9*. Moreover, when oocytes were depleted in adult female mice using an inducible form of the human Diphtheria toxin receptor (hDTR), which only induces oocyte-specific cell ablation after administration of diphtheria toxin (*Gdf9:Cre; R26iDTR*), *Sox9* expression was also not detected (Uhlenhaut et al., 2009). Again these results differ from the ones obtained with the conditional adult XX $Foxl2^{\Delta/\Delta}$ mice, which showed an up-regulation of *Sox9* expression at a comparable time after FOXL2 depletion.

Taken together, *Sox9* de-repression could not be detected in XX gonads upon oocyte depletion - it occurs only upon loss of *Foxl2*. Moreover, oocytes could

still be found in the ovaries of both XX *Foxl2*$^{LacZ/LacZ}$ and *Foxl2*$^{\Delta/\Delta}$ mice. These results indicate that the de-repression of *Sox9* in XX gonads is a consequence of loss of *Foxl2* expression, which results in a transdifferentiation of granulosa cells into Sertoli-like cells and that oocytes are not directly required to maintain the granulosa cell fate at least during postnatal ovarian development. The loss of oocytes in *Foxl2* mutants might be a consequence of the granulosa cell transdifferentiation to Sertoli-like cells rather than a cause. It is not surprising that oocytes fail to survive in a testicular environment.

In summary, *Foxl2* seems to be the only critical factor ensuring the maintenance of the granulosa cell lineage in the adult ovary. It does so by repressing *Sox9* expression via the TESCO element. However, these data also indicate that FOXL2 is not the primary factor to repress *Sox9* expression during embryonic development. Thus, other factors must be involved to ensure both the establishment and maintenance of the female supporting precursor cell during embryogenesis.

Chapter 6

The effect of *Wnt4* on TESCO activity *in vivo*

6.1 Introduction

Loss of *Foxl2* expression results in the de-repression of TESCO activity and endogenous *Sox9* expression after birth, indicating that FOXL2 is a critical factor for maintaining granulosa cell fate postnatally (Chapter 5 and Uhlenhaut et al. (2009)). However, *Foxl2* does not seem to be the important factor to repress both TESCO and *Sox9* during embryogenesis. This raises the question which factor(s) are responsible for the initial down-regulation of *Sox9* expression in the XX gonad at 11.5 dpc and maintenance of this repression throughout embryonic development.

Wnt4 is a candidate to fulfil this role. In the mouse, *Wnt4* is expressed in the indifferent gonad from 10.5 dpc onwards in both sexes and becomes female-specific due to its down-regulation in XY gonads at 11.5 dpc (Vainio et al., 1999). XX

mice carrying a targeted null mutation of *Wnt4* (*Wnt4*$^{-/-}$) show partial masculinisation of the gonad (which takes on a rounder shape, is unencapsulated and develops closely associated with a fat body) and the developing gonadal duct has a region resembling the epididymal region of the male Wolffian duct. Leydig-like cells, expressing the two male steroidogenic genes 3β*HSD* and *P450c17* are present in the mutant XX gonad and produce testosterone (Vainio et al., 1999). Microarray studies revealed that several genes involved in testosterone synthesis are up-regulated in XX *Wnt4*$^{-/-}$ gonads between 12.5 dpc and 14.5 dpc (Heikkilä et al., 2005). In these mice, steroidogenic adrenal precursors, derived from the mesonephric region where the adrenal is forming, migrate into the gonad and cluster in the area closest to the developing adrenal. This lead to the hypothesis that during early gonadal development in XX mice, WNT4 represses the migration of steroidogenic cells from the mesonephros into the gonad (Jeays-Ward et al., 2003). Moreover, mesonephric cells, which in XY gonads contribute to the formation of the male-specific blood vessel, migrate into XX *Wnt4*$^{-/-}$ gonads and an ectopic coelomic vessel develops. This suggests that *Wnt4* is necessary to prevent both migration of endothelial cells into the XX gonad as well as formation of the vessel (Jeays-Ward et al., 2003). However, neither testis cord formation nor masculinisation of the external genitalia could be observed in XX *Wnt4*$^{-/-}$ mice. Moreover, while *Sox9* expression could be detected transiently in these mutant gonads at 11.5 dpc, it was already down-regulated by 12.5 dpc (Kim et al., 2006b). Lack of *Wnt4* expression also results in loss of germ cells by the time of birth. While the early migration and proliferation of the germ cells seem to be normal in XX *Wnt4*$^{-/-}$ mice, almost 90% of the germ cells undergo apopto-

sis after 16.5 dpc (Yao et al., 2004), indicating a role for Wnt4 in maintaining oocyte health. Interestingly, XY *Wnt4*$^{-/-}$ mice display defects in early testis development, including reduced expression of *Sox9*, *Dhh* and *Amh* at 11.5 dpc (Jeays-Ward et al., 2004). As *Sry* expression is unaffected, this data suggests that *Wnt4* is involved in Sertoli cell differentiation downstream of *Sry* but either upstream of or in parallel to *Sox9*. WNT4 has a mutually antagonistic relationship with FGF9 (Kim et al., 2006b) and it is possible that precocious activity of the latter leads to precocious development of testis cords with too few Sertoli-cells dividing more slowly than pre-Sertoli cells. XY *Wnt4*$^{-/-}$ gonads also exhibit an increase in the number of steroidogenic cells indicating that *Wnt4* might play a regulatory role in steroidogenesis in both XX and XY gonads (Jeays-Ward et al., 2004).

XX mice carrying double homozygous mutations in *Wnt4* and *Foxl2* (*Foxl2*$^{-/-}$, *Wnt4*$^{-/-}$) develop some seminiferous tubule-like structures at P0, which contain oocytes as well as differentiated type A spermatogonia (Ottolenghi et al., 2007). Several male-specific marker genes such as *Sox9*, *Dmrt1*, *Amh*, *Ptgds*, *Hsd17b3* and *Cyp26b1* are up-regulated in XX *Foxl2*$^{-/-}$, *Wnt4*$^{-/-}$ gonads just after birth (Ottolenghi et al., 2007).

In contrast to the data from *WNT4* duplications in human patients, misexpression of either the human *WNT4* gene with its endogenous regulatory sequences or an Sf1:Wnt4 construct in XY transgenic mice does not cause male-to-female sex reversal. In the XY *Wnt4* transgenic gonads, the coelomic blood vessel develops, although it is disorganised with multiple branches and fails to form one

main vessel. This suggests that WNT4 is not sufficient to prevent the formation of the coelomic blood vessel in XY gonads, although the level and timing of *Wnt4* expression may not have been sufficient (Jeays-Ward et al., 2003; Jordan et al., 2003). These data not only imply that additional *Wnt4*-independent pathways must exist in XX gonads to prevent the initiation of vessel formation but also that *Wnt4* needs to be adequately down-regulated in XY gonads in order to form normal vessels. While misexpression Sf1:Wnt4 in XY gonads does not affect Leydig cell differentiation (Jeays-Ward et al., 2003), the presence of human WNT4 results in reduced levels of testosterone due to a decreased expression of *StAR*, which is involved in the rate-limiting step of steroidogenesis (Jordan et al., 2003).

In XY gonads, *Fgf9* has been shown to be involved in the maintenance of *Sox9* expression via the establishment of a feed-back loop between SOX9 and FGF9 (Kim et al., 2006b). In XY $Fgf9^{-/-}$ gonads, *Wnt4* expression is not down-regulated at 11.5 dpc as it normally occurs. However, both *Sry* and *Sox9* are initially expressed. Furthermore, addition of FGF9 to XX gonad cultures *ex vivo* resulted in the down-regulation of *Wnt4* expression (Kim et al., 2006b), while *Fgf9*, which is normally expressed only in XY gonads after 11.5 dpc, was detected in XX $Wnt4^{-/-}$ gonads at 12.5 dpc (Kim et al., 2006b). These results suggest an antagonistic relationship between FGF9 and WNT4 signalling in the developing gonad: the establishment of active FGF9 signalling in the XY gonad represses *Wnt4* expression and thereby ensures normal testis development, whereas the establishment of WNT4 signalling represses *Fgf9* activity in XX gonads to secure normal ovarian development. In XX gonads with ectopic *Sry* expression induced after the critical time window of sex determination, *Sox9* expression could not

be maintained. This was attributed to the fact that delayed *Sry* expression can not result in sufficiently high levels of *Sox9* expression to enable the XX gonad to activate FGF9 signalling. As the XX gonads did not switch from the female WNT4 signalling to the male FGF9 signalling program, *Sox9* expression could not be maintained and therefore no female-to-male sex reversal occurred (Hiramatsu et al., 2009). Taken together, the establishment of WNT4 signalling seems to be crucial in tipping the balance of sex determination towards female development.

So far, a number of genes have been described as possible downstream targets for WNT4 signalling. For example *Dax1* expression is significantly reduced in $Wnt4^{-/-}$ mice (Mizusaki et al., 2003). Moreover, addition of WNT4 to cultured mouse Leydig cells or Sertoli cells resulted in an up-regulation of endogenous *Dax1* expression (Jordan et al., 2001). Furthermore, both *Fst* and *Bmp2* have been suggested as downstream effectors of WNT4 signalling. *Fst* encodes an activin binding protein, which can antagonise members of the TGFβ superfamily. It was originally identified as a potent inhibitor of pituitary FSH (Ueno et al., 1987) and is involved in regulation of the hypothalamic-pituitary-gonadal axis (Phillips and de Kretser, 1998). *Fst* is expressed exclusively in XX gonads from 11.5 dpc and decreases after 14.5 dpc (Yao et al., 2004). In XX $Wnt4^{-/-}$ mice, no expression of *Fst* can be detected, whereas *Wnt4* is expressed normally in XX $Fst^{-/-}$ mice. This suggests that *Fst* is a downstream effector of Wnt4 signalling (Yao et al., 2004). Moreover, XX $Fst^{-/-}$ mice form testis-specific coelomic blood vessels and suffer germ cell depletion similar to the phenotypes observed in $Wnt4^{-/-}$ mice (Yao et al., 2004). Testis differentiation was found to be normal in XY $Fst^{-/-}$ mice. BMP2 belongs to the family of bone morphogenic proteins

(Bmp), which are multifunctional regulators of cell growth and differentiation and were originally identified in cartilage formation (Wozney et al., 1988). *Bmp2* is expressed in the gonads of both sexes at 10.5 dpc and becomes female-specific around 11.5 dpc, before its expression decreases after 14.5 dpc (Yao et al., 2004). The expression is restricted to cells just under the coelomic epithelium. The gonadal phenotype of *Bmp2*$^{-/-}$ mice could not be analysed as the mice die before 10.5 dpc and thus prior to the time of sex determination (Zhang and Bradley, 1996). However, *Bmp2* expression is absent in *Wnt4*$^{-/-}$ mice suggesting that it is a downstream effector of Wnt4 signalling (Yao et al., 2004).

In summary, WNT4 is an important anti-testis factor, implicated in opposing the male pathway by repressing SOX9 and FGF9. This raises the question whether *Wnt4* could be involved in the regulation of *Sox9* expression *in vivo* via the TESCO enhancer element. To investigate this possibility, the effect of *Wnt4* deletion on TESCO activity was analysed in XX gonads by crossing *Wnt4* mutant mice to the TESCO:CFP reporter line. If WNT4 represses *Sox9* expression via the TESCO enhancer element *in vivo*, loss of *Wnt4* in the XX gonad should result in a de-repression of TESCO activity and therefore activation of CFP expression in XX *TESCO:CFP; Wnt4*$^{-/-}$ ovaries.

6.2 Results

6.2.1 The de-repression of TESCO in *Wnt4* mutant mice

To test whether TESCO became de-repressed in the developing gonad upon loss of *Wnt4* expression, the TESCO:CFP reporter mice were bred to *Wnt4* mutant mice. The targeted deletion of *Wnt4* was established by replacing the third and fourth exon of *Wnt4* with a selection cassette containing neomycin phosphotransferase resulting in a functional null allele (Stark et al., 1994).

Wnt4$^{-/-}$ mice die within 24 hours after birth due to kidney failure and therefore no analyses were possible after P0 (Vainio et al., 1999). XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads, as well as XY *TESCO:CFP; Wnt4*$^{+/-}$ and XX *TESCO:CFP; Wnt4*$^{+/-}$ control gonads, were collected at different time points during embryonic development (12.5 dpc to 18.5 dpc) and at birth (P0). Gonads were then sectioned and analysed for CFP expression. CFP expression was detected in XY *TESCO:CFP; Wnt4*$^{+/-}$ gonads in the Sertoli cells. In XX *TESCO:CFP; Wnt4*$^{+/-}$ gonads, occasionally a few CFP-positive cells could be detected from 14.5 dpc onwards. In XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads, few CFP expressing cells were detected at 14.5 dpc, while more CFP-positive cells could be found between 16.5 dpc and P0 (Figure 6.1).

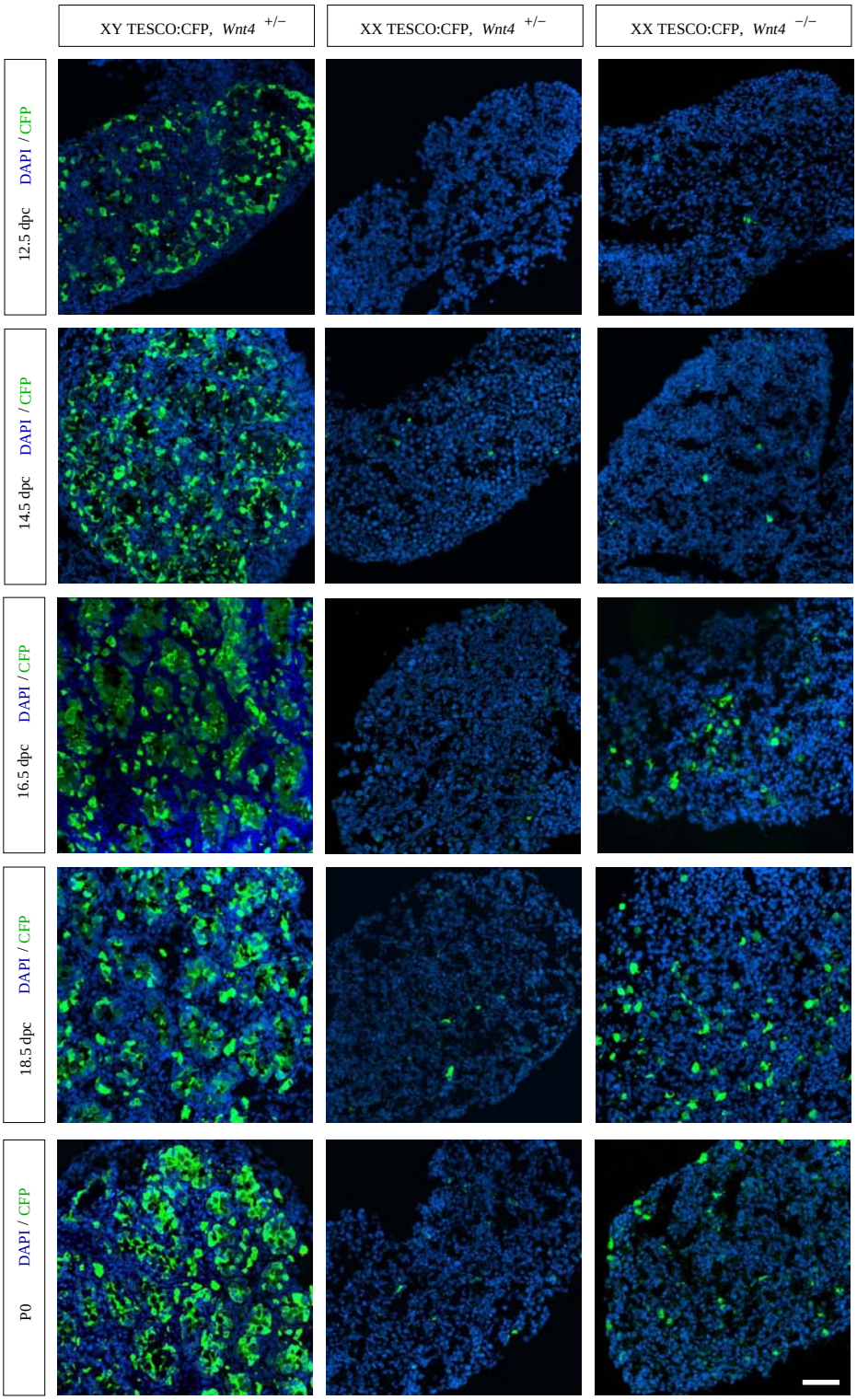


Figure 6.1: **TESCO activity in *TESCO:CFP; Wnt4*$^{-/-}$ gonads during embryogenesis.**
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of *Wnt4* at different time points during embryogenesis and just after birth (P0). Immunostaining shows DAPI in blue and CFP in green. TESCO expression could be detected in the Sertoli cells in XY gonads (left panel). Very few CFP-positive cells could be found in XX *TESCO:CFP; Wnt4*$^{+/-}$ gonads (middle panel). De-repression of TESCO activity was found in XX *TESCO:CFP; Wnt4*$^{-/-}$ from around 14.5 dpc to P0 (right panel). Scale bar = 50μm.

To determine whether the TESCO element was de-repressed in the granulosa cells of XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads, co-immunostainings of CFP and FOXL2 were analysed in 18.5 dpc gonads. As expected, XY *TESCO:CFP; Wnt4*$^{+/-}$ gonads expressed CFP, as shown before, but no FOXL2. On the other hand, XX *TESCO:CFP; Wnt4*$^{+/-}$ gonads showed expression of FOXL2 and occasionally very few CFP-positive cells. Expression of both proteins was detected in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads. Although both proteins were expressed, strong signals for FOXL2 and CFP did not co-localise in the mutant XX gonads (Figure 6.2). Only occasionally a very weak cytoplasmic CFP signal could be found with a strong nuclear FOXL2 signal and vice versa.

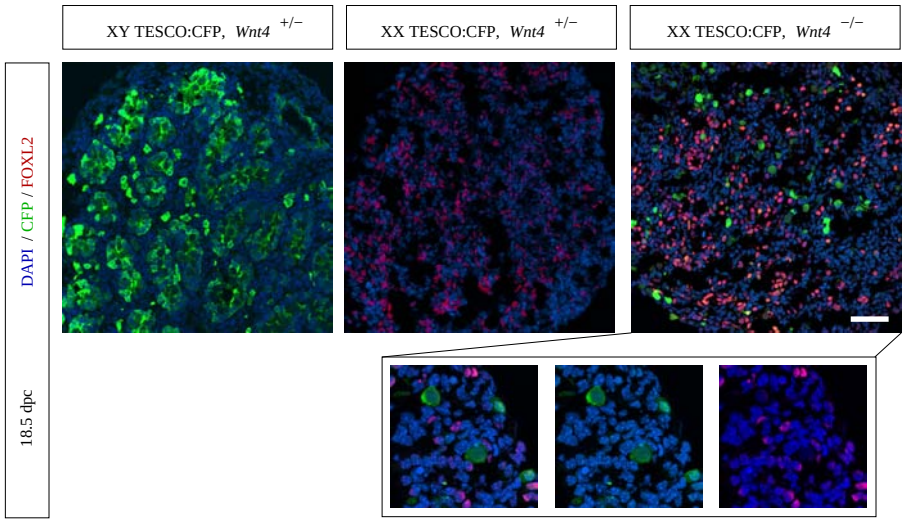


Figure 6.2: **FOXL2 and CFP do not co-localise in *TESCO:CFP; Wnt4*$^{-/-}$ gonads.**
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of *Wnt4* at 18.5 dpc. Immunostaining showing DAPI in blue, CFP in green and FOXL2 in red. CFP was detected in the Sertoli cells in the XY gonads and occasionally in scattered cells in the XX *TESCO:CFP; Wnt4*$^{+/-}$ gonads, whereas FOXL2 was detected in XX *TESCO:CFP; Wnt4*$^{+/-}$ but not XY gonads. XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads showed cells which express either CFP or FOXL2. Scale bar = 50μm; inserts show 2.5x higher magnifications.

6.2.2 SOXE expression in *Wnt4* mutant mice

Next, the expression pattern of endogenous *Sox9* was analysed between 12.5 dpc and P0 to determine if it coincided with the CFP expression. As expected, *Sox9* expression was detected in Sertoli cells of XY *TESCO:CFP; Wnt4*$^{+/-}$ gonads, while it could not be detected in control XX *TESCO:CFP; Wnt4*$^{+/-}$ gonads. However, no *Sox9* expression was found in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads between 12.5 dpc and P0 (Figure 6.3). Thus, the cells which express TESCO:CFP did not show a synchronous de-repression of endogenous *Sox9* expression.

It has been described before that other members of the SoxE family also play an important role in early testis development. *Sox8* is expressed in Sertoli cells in the XY gonad from 12.0 dpc onwards and has not been detected in XX gonads (Schepers et al., 2003). Recently, it has also been reported that *Sox10* is expressed exclusively in the XY gonad at the time of sex determination (Polanco et al., 2010). Both, *Sox8* and *Sox10* share a high sequence identity with *Sox9* and it has been proposed that at least SOX8 and SOX9 might act redundantly with respect to each others function. To determine whether any SOXE signal could be detected in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads, an antibody recognising all three members of the SOXE proteins (SOX8, SOX9, SOX10) was used.

Since SOX9 expression is absent in XX *Wnt4*$^{-/-}$ gonads (Figure 6.3) any SOXE signal identified would correspond to SOX8 or SOX10. As expected, SOXE expression was found in the Sertoli cells in XY *TESCO:CFP; Wnt4*$^{+/-}$ gonads at all time points analysed, presumably representing the expression patterns of both *Sox8* and *Sox9* (Figure 6.4). No SOXE signal was detected in control XX

TESCO:CFP; Wnt4$^{+/-}$ gonads. In XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads, some occasional SOXE-positive cells could be found at 12.5 dpc, a few more cells at 14.5 dpc and more cells expressing SOXE were detected between 16.5 dpc and P0 (Figure 6.4), similar to the pattern of TESCO de-repression in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads (Figure 6.1).

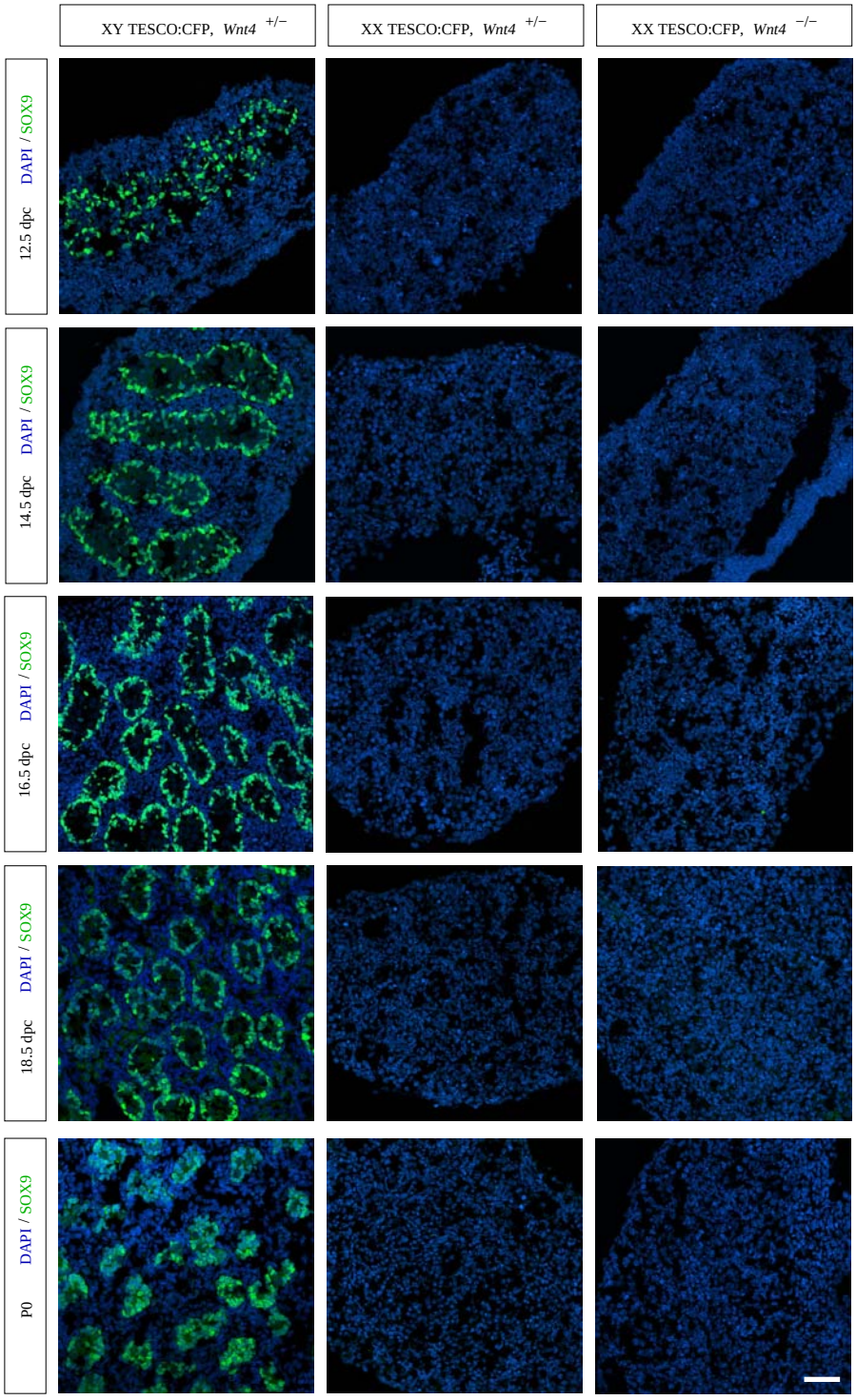


Figure 6.3: **Endogenous *Sox9* expression in *TESCO:CFP; Wnt4*$^{-/-}$ gonads during embryogenesis.**
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of *Wnt4* at different time points during embryogenesis and just after birth (P0). Immunostaining shows DAPI in blue and SOX9 in green. SOX9 could be detected in Sertoli cells in the XY *TESCO:CFP; Wnt4*$^{+/-}$ gonads (left panel) but not in XX *TESCO:CFP; Wnt4*$^{+/-}$ or XX *TESCO:CFP; Wnt4*$^{-/-}$ at any time during embryogenesis (middle and right column, respectively). Scale bar = 50μm.

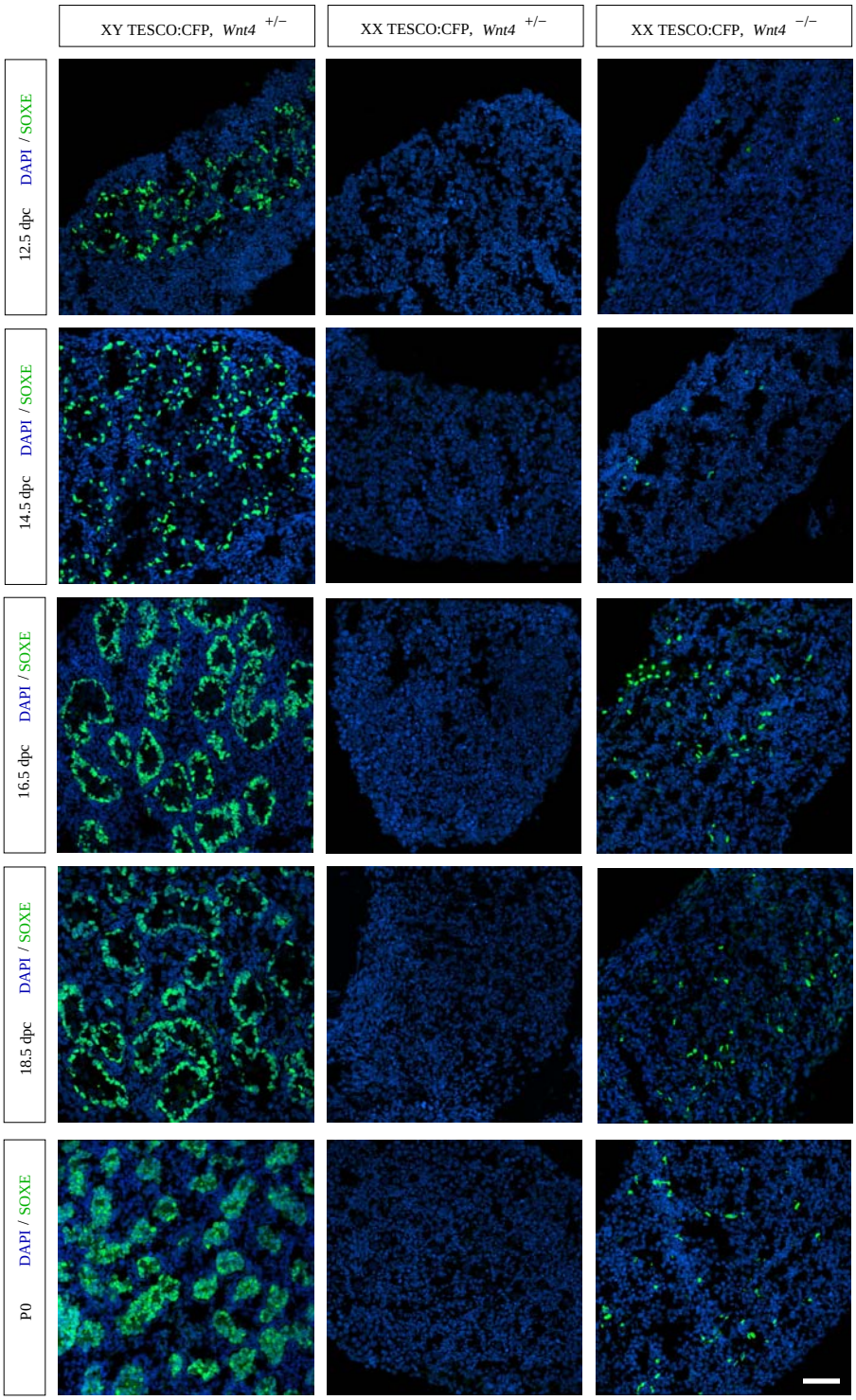


Figure 6.4: **Endogenous *SoxE* expression in *TESCO:CFP; Wnt4*$^{-/-}$ gonads during embryogenesis.**
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of *Wnt4* at different time points during embryogenesis and at P0. Immunostaining shows DAPI in blue and SOXE in green. SOXE signals could be found in Sertoli cells in the XY *TESCO:CFP; Wnt4*$^{+/-}$ gonads (left panel) but not in XX *TESCO:CFP; Wnt4*$^{+/-}$ gonads (middle panel). De-repression of SOXE, presumably SOX8 and/or SOX10, was found in XX *TESCO:CFP; Wnt4*$^{-/-}$ from around 14.5 dpc until birth (right panel). Scale bar = 50μm.

6.2.3 De-repression of TESCO and endogenous *Sox9* expression in *Foxl2/Wnt4* double mutant mice

Next, the effect of the combined loss of both *Foxl2* and *Wnt4* on TESCO activity was analysed in gonads of XX *Foxl2/Wnt4* double mutant mice ($Foxl2^{LacZ/LacZ}$; $Wnt4^{-/-}$) at birth (Figure 6.5). CFP expression could be detected normally in Sertoli cells in XY $Foxl2^{LacZ/LacZ}$; $Wnt4^{-/-}$ gonads, indicating that both *Foxl2* and *Wnt4* are not critical for Sertoli cell differentiation or testis cord formation. Analyses of XX *TESCO:CFP;* $Foxl2^{LacZ/LacZ}$*;* $Wnt4^{+/-}$ gonads showed similar results to those of heterozygous XX *TESCO:CFP;* $Wnt4^{+/-}$ single mutants with a few CFP-positive cells. However, XX *TESCO:CFP;* $Foxl2^{LacZ/LacZ}$*;* $Wnt4^{-/-}$ gonads showed an increase in the number of CFP-positive cells compared to each single mutant, suggesting a stronger de-repression of TESCO activity at P0 in the $Foxl2^{LacZ/LacZ}$; $Wnt4^{-/-}$ double mutants. The CFP-positive cells in these gonads are forming seminiferous tubule-like structures which resemble the ones seen in XY gonads. Endogenous SOX9 expression could be found in the Sertoli cells of XY $Foxl2^{LacZ/LacZ}$; $Wnt4^{-/-}$ gonads, but not in the analysed XX *TESCO:CFP;* $Foxl2^{LacZ/LacZ}$*;* $Wnt4^{+/-}$ gonads. XX *TESCO:CFP;* $Foxl2^{LacZ/LacZ}$*;* $Wnt4^{-/-}$ gonads showed endogenous *Sox9* expression in a pattern similar to the TESCO:CFP reporter. This was in contrast to each single mutant, where SOX9 was not de-repressed at P0 (Figure 5.5 and Figure 6.3).

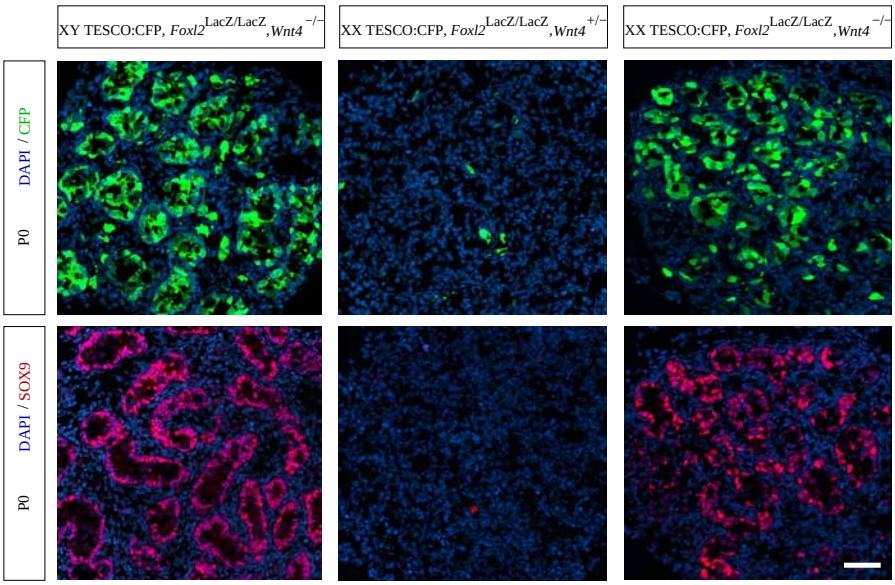


Figure 6.5: **TESCO activity in *TESCO:CFP; Foxl2$^{LacZ/LacZ}$; Wnt4$^{-/-}$* double mutant gonads at P0.**
Cryosections of XY and XX gonads of TESCO:CFP mice with homozygous loss of *Foxl2* and heterozygous or homozygous loss of *Wnt4* at P0. Immunostaining shows DAPI in blue and CFP in green. TESCO expression could be detected in the Sertoli cells in the XY *TESCO:CFP; Foxl2$^{LacZ/LacZ}$; Wnt4$^{-/-}$* gonads. Very few CFP-positive cells could be found in XX *TESCO:CFP; Foxl2$^{LacZ/LacZ}$; Wnt4$^{+/-}$* gonads. More CFP-positive cells were found in XX *TESCO:CFP; Foxl2$^{LacZ/LacZ}$; Wnt4$^{-/-}$* gonads than in the single mutants. Scale bar = 50μm.

6.3 Conclusions

According to published data, *Wnt4* is a candidate for an anti-testis or ovarian-determining gene which could be involved in the repression of *Sox9* in XX gonads during embryonic development. As *Wnt4* expression becomes female-specific coincidentally with the down-regulation of *Sox9* at 11.5 dpc, it is possible that it is the crucial gene to mediate the down-regulation of *Sox9* expression in embryonic XX gonads. In this chapter it was analysed whether *Wnt4* can repress *Sox9* expression *in vivo* and whether this repression is mediated via the TESCO enhancer element. As homozygous *Wnt4* mutant mice die within 24 h after birth due to kidney failure, the gonadal phenotype of these mice could only be analysed up until P0.

Homozygous loss of *Wnt4* resulted in some de-repression of TESCO activity in the XX gonad around 14.5 dpc. This result suggests that WNT4 signalling is involved in the repression of TESCO activity from 14.5 dpc until after birth, but not prior to 14.5 dpc. Interestingly, no *Sox9* expression could be detected in XX $Wnt4^{-/-}$ gonads at the time points analysed, either during embryogenesis or at P0. This is consistent with results from Kim et al. (2006b), where transient expression of *Sox9* in XX $Wnt4^{-/-}$ gonads was detected only at 11.5 dpc and then found to be down-regulated by 12.5 dpc. The time course of *Sox9* expression in XX *TESCO:CFP;* $Wnt4^{-/-}$ gonads shown here, demonstrates that the down-regulation of *Sox9* is maintained after 12.5 dpc until birth, although some cells express the TESCO:CFP reporter from 14.5 dpc onwards. The fact that the TESCO element became de-repressed upon loss of *Wnt4* while *Sox9* expression is

not up-regulated suggests that the endogenous *Sox9* gene is controlled in a more complex manner, independently of the TESCO element in XX gonads during embryonic development. The TESCO element is the critical 1.3 kb core region of the endogenous 3.2 kb *Sox9* enhancer TES and thus it might be possible that the complete TES element contains relevant repressor binding sites outside the TESCO region. In fact, when the larger TES element was cloned into the luciferase vector and tested in co-transfection assays, the element could not be activated by increasing amounts of SF1 and SOX9 (data not shown), suggesting that TES might contain a strong repressor element outside TESCO. However, as mentioned before, TESCO:CFP is a transgene and it is possible that the differential de-repression profile of TESCO:CFP transgene and endogenous *Sox9* might be at least partially due to epigenetic differences and integration site.

Coinciding with the de-repression of TESCO in XX $Wnt4^{-/-}$ gonads, was the activation of SOXE expression, that is SOX8 and/or SOX10, from 14.5 dpc until P0. This result suggests that WNT4 represses *Sox8* and/or *Sox10* expression, in addition to *Sox9*, in XX gonads during this time of embryonic development. Both, *Sox8* and *Sox10* belong to the same group of SOXE proteins as *Sox9* and the two proteins share a high sequence homology. Studies in mice with double mutation of both *Sox8* and *Sox9* have suggested that the two genes have redundant functions during embryonic testis development (Chaboissier et al., 2004; Barrionuevo et al., 2009).

Interestingly, the cells which showed a de-repression of TESCO activity in XX $Wnt4^{-/-}$ gonads were not expressing FOXL2. This suggests that TESCO ac-

tivity becomes de-repressed in a subset of granulosa cells which have lost *Foxl2* expression, or in some undifferentiated granulosa precursor cells in the gonads of these mice. The result that expression of TESCO and FOXL2 are mutually exclusive, once more indicates that FOXL2 antagonises TESCO activity. This is consistent with the results in adult XX $Foxl2^{\Delta/\Delta}$ mice, were the expression of FOXL2 and SOX9 were found to be mutually exclusive (Uhlenhaut et al., 2009). FOXL2 seems to be able to keep TESCO activity repressed in the granulosa cell precursor cells in XX $Wnt4^{-/-}$ gonads during embryonic development. As TESCO does not become up-regulated in XX $Foxl2^{LacZ/LacZ}$ gonads before birth, it suggests that *Wnt4* is the more important gene to repress TESCO during embryogenesis, but in the absence of *Wnt4*, FOXL2 can still repress TESCO activity. To determine whether *Foxl2* and *Wnt4* might be acting in redundant pathways to secure repression of TESCO activity and endogenous *Sox9* expression in the developing XX gonad, mice carrying mutations of both genes were analysed. XX $Foxl2^{LacZ/LacZ}$, $Wnt4^{+/-}$ gonads were similar to those of $Wnt4^{+/-}$ mice with very few CFP-positive cells. XX $Foxl2^{LacZ/LacZ}$, $Wnt4^{-/-}$ gonads showed more cells expressing CFP than in either of the single mutants. Moreover, cord-like structures could be seen in the double mutant gonads and *Sox9* expression was de-repressed. This observation was different from that in each single mutant where neither XX $Foxl2^{LacZ/LacZ}$ nor XX $Wnt4^{-/-}$ gonads showed up-regulation of *Sox9* expression at P0 (Figure 6.6). These results indicate a synergistic effect of both *Foxl2* and *Wnt4* in repressing TESCO and *Sox9* expression at birth.

Taken together these results indicate that WNT4 signalling is at least partially involved in the repression of TESCO activity during embryonic development.

However, the expression of endogenous *Sox9* seems to be repressed by additional factors independently from the TESCO element. Around the time of birth both *Wnt4* and *Foxl2* seem to be working together to ensure the repression of TESCO activity and *Sox9* expression in the developing XX gonad.

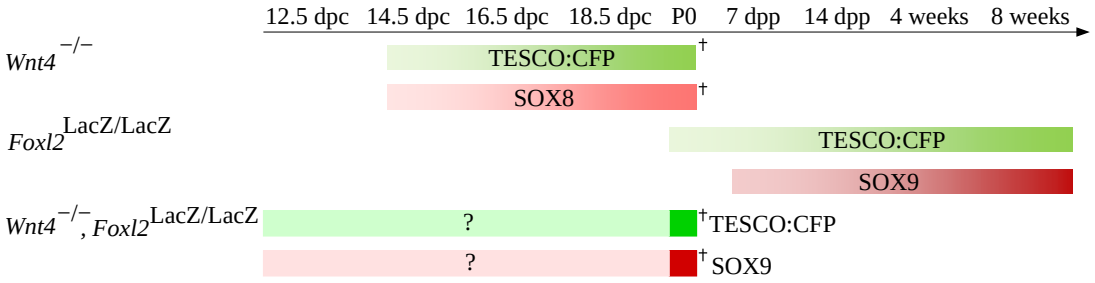


Figure 6.6: **Summary of *in vivo* data.**
In gonads of XX $Wnt4^{-/-}$ mice, TESCO:CFP and SOX8 are de-repressed from 14.5 dpc until P0, while *Sox9* is still repressed (cross indicates that $Wnt4^{-/-}$ mice die within 24h after birth). In gonads of XX $Foxl2^{LacZ/LacZ}$ mice, TESCO:CFP becomes de-repressed at P0, whereas SOX9 first appears at 7 dpp. In gonads of XX double mutants, both TESCO:CFP and SOX9 can be detected at P0.

Chapter 7

General Discussion

The decision whether a mammalian embryo develops as male or female depends on the presence or absence of the Y chromosome. For some, the discovery of *Sry* as the sex determining factor on the Y almost 20 years ago, strengthened the idea that male development would be an active process whereas female development would be merely a default pathway. However, due to the discovery of a number of genes involved in both male and female sex determination, it has become evident that it is not that simple. In both humans and mice, cases of XX female-to-male sex reversal have been described that result from LoF mutations in several genes, which are now considered to be ovarian-promoting or at least anti-testis genes as they are playing important roles in the active repression of the male pathway.

Recently, it has been shown that *Sox9* is the critical downstream target gene of *Sry* in the developing XY gonad and that its testis-specific expression is regulated via the 1.3 kb enhancer element TESCO (Sekido and Lovell-Badge, 2008). A model has been proposed in which TESCO activity (and therefore *Sox9* expression) in the XY supporting cell lineage is initiated by SF1 at 10.5 dpc, up-

regulated by SRY by 11.5 dpc and maintained via a positive feedback loop including SOX9 itself and other factors, such as FGF9 and PGD_2 signalling (Sekido and Lovell-Badge, 2008; Kim et al., 2006b; Wilhelm et al., 2005). SOX9 directs the supporting cell lineage of the XY gonad to become Sertoli cells, which is the crucial event in testis development. In contrast, in XX gonads, although *Sox9* expression is initiated by SF1, it is then actively down-regulated by 11.5 dpc and this repression is maintained in granulosa cells usually throughout life. Until now, it has not been known which genes are responsible for the initial down-regulation of *Sox9* and the maintenance of *Sox9* repression or whether this repression is mediated via the TESCO element in XX gonads.

7.1 The ovarian-promoting genes *Dax1*, *Sox4* and *Foxl2* repress TESCO activity *in vitro*

Several genes have been proposed as ovarian promoting genes due to their differential expression profiles in XX and XY gonads during the time of sex determination. These include *Dax1*, *Sox4*, *Wnt4*, *Foxl2* and *ERα*. To analyse whether these candidate genes could be involved in the regulation of *Sox9* expression via the TESCO element, the 1.3 kb sequence was first searched for putative binding sites. The analysis revealed that the TESCO sequence is indeed full of potential transcription factor binding sites. Amongst others, sites were found for GATA, zinc finger transcription factors, LIM homeobox factors, TATA-binding factors, SOX proteins, nuclear receptors, LEF/TCFs, forkhead factors and estrogen response elements. This suggests the possibility that the listed ovarian promoting

genes could be involved in the modulation of TESCO activity.

The potential of these genes to modulate *Sox9* expression via the TESCO element was then analysed in co-transfection assays *in vitro*. In this thesis, it has been demonstrated that, while WNT effectors did not significantly affect TESCO activation mediated by SF1 and SOX9/SRY, DAX1, SOX4 and FOXL2 were able to repress TESCO activity in a dosage-dependent manner. Interestingly, ERα on its own did not have any effect on TESCO activation, but acted synergistically with FOXL2 resulting in an enhanced repression effect which seems to be independent from the amount of ERα, at least at the levels tested.

The implication of WNT4 signalling on TESCO expression was analysed via its downstream effectors β-catenin and LEF/TCFs. No effect on TESCO activity could be found by either β-catenin on its own, either LEF1, TCF1, TCF3 or TCF4, or by combinations of β-catenin and any of the LEF/TCFs. This could suggest that WNT4 signalling is not acting on *Sox9* expression via the TESCO element or that it acts on TESCO activity via a non-canonical pathway (for review see Komiya and Habas, 2008). Another possibility is that the COS7 cell line that was used might lack important co-factors which are crucial for WNT dependent transcriptional regulation (like Groucho or CtBP). For more conclusive results the experiments should be repeated in a more suitable cell line, e.g. in primary cultures of granulosa cells. It is not known yet which LEF/TCFs are expressed in the supporting cells of the gonad and therefore could be involved in any regulation of sex determination. Thus, in future experiments, it should firstly be determined which LEF/TCFs are indeed expressed in the granulosa cells in XX gonads during

embryogenesis and postnatally, and thus could be involved in the regulation of *Sox9* expression. Moreover, ChIP assays are necessary to analyse which of these factors, if any, are bound to the TESCO element *in vivo* and thus might indeed be involved in its regulation. It is also possible that WNT4 signalling modulates *Sox9* expression not by transcriptional regulation but via a different mechanism. It has been reported in chondrocyte differentiation that SOX9 physically interacts with β-catenin which causes the degradation of both proteins via the proteosomal machinery (Akiyama et al., 2004). It might be possible that the same is happening in the gonad, thereby regulating the amount of available SOX9. Nevertheless, reduced levels of SOX9 in the presence of β-catenin should still cause a reduction of TESCO activation in the co-transfection assays, as SOX9 is needed for TESCO activation. However, the luciferase reporter assay might not be a sufficient system to analyse any potential SOX9 degradation effect. To determine the effect of β-catenin on SOX9, different amounts of β-catenin and SOX9 could be transfected into a cell line which does not endogenously express both genes and a possible reduction of the two proteins could be monitored e.g. by western blotting analysis. Finally, it is also possible that WNT4 is only involved in the regulation of *Sox9* expression via activating its downstream target *Dax1* (Mizusaki et al., 2003), which is unlikely to happen in COS7 cells.

In the co-transfection assays, DAX1 was able to repress TESCO activation. It had already been reported that DAX1 can form heterodimers with SF1 and decreases SF1 activity (Ito et al., 1997; Crawford et al., 1998). As SF1 is needed for TESCO activation, less SF1 activity could result in the observed repression effect on TESCO activity upon addition of DAX1. However, the precise mechanism as

to how DAX1 represses TESCO activity remains to be investigated. In future experiments it would be necessary to determine whether DAX1 directly binds to the TESCO sequence, e.g. by ChIP assay. Moreover, it would also be interesting to analyse the effect of DAX1 on TESCO activity *in vivo*. If DAX1 is necessary for the repression of TESCO activity, mice carrying a deletion of the *Dax1* gene crossed to the TESCO:CFP reporter line might show an up-regulation of CFP expression in XX gonads. However, the role of *Dax1* in sex determination is not completely understood and the published *Dax1* mutant mice are still a point of controversy. Thus, it might be necessary to generate a targeted deletion of both *Dax1* exons or to establish a complete knock down of *Dax1* expression via other methods such as RNAi. These *in vivo* models might provide a more reliable model to analyse the effect of loss of *Dax1* expression in general and on *Sox9* expression and TESCO activation in particular.

SOX4 was also found to be able to repress TESCO activity *in vitro*, but only when activated by combinations including SOX9 or SRY. The SOX4 protein contains the highly conserved DNA binding domain (HMG-box) characteristic of the SOX proteins and it is likely that it can bind to the same SOX binding sites in the TESCO element as SOX9 and SRY. Thus, SOX4 could compete with SOX9 and/or SRY for binding to the TESCO element and thereby prevent the activation of TESCO. As SOX4 and SOX9 are members of different groups of SOX proteins it is also possible that SOX9 (belonging to the SOXE group) acts as a transcriptional activator on TESCO and SOX4 (belonging to the SOXC group) could act as a transcriptional repressor. Indeed, it has already been shown that a prolonged expression of *Sox4* in oligodendrocytes results in a reduction of

myelin gene expression *in vivo* (Chew and Gallo, 2009). In future experiments, such as EMSA or ChIP assays, it should be determined whether SOX4 is indeed able to bind to the SOX binding sites in the TESCO element and whether this might result in a competition for binding with SOX9. Furthermore, the effect of SOX4 on TESCO activity and endogenous *Sox9* expression should be analysed *in vivo*. For this, mice with a deletion of the *Sox4* gene should be crossed to the TESCO:CFP reporter line. If SOX4 is repressing TESCO activation, homozygous loss of *Sox4* might result in an up-regulation of CFP and possibly endogenous *Sox9* expression in XX gonads. However, $Sox4^{-/-}$ mice die at 14.5 dpc due to heart malformation (Schilham et al., 1996) allowing only the analysis of a possible role of SOX4 in the early repression of *Sox9*. To further determine the involvement of SOX4 in *Sox9* repression after 14.5 dpc, a conditional mutation of *Sox4* would be required.

The most interesting effect seen in the co-transfection assays *in vitro* was the repression of TESCO by FOXL2. This effect was dosage-dependent and provides the first evidence of a direct link between *Foxl2* and the regulation of *Sox9*, suggesting that this regulation could be mediated via the TESCO element.

7.2 FOXL2 represses TESCO activity synergistically with ERα

FOXL2 and ERα were able to synergistically repress TESCO activation *in vitro*. While FOXL2 belongs to the forkhead family of transcription factors which can bind to DNA at the consensus binding site (A/G)(C/T)(A/C)AA(A/T)A (Kauf-

mann et al., 1995), ERα can bind to EREs (palindromes of GGTCA) (Klinge, 2001), both of which are present in the TESCO element. ChIP analysis in adult ovaries have revealed that both FOXL2 and ERα are indeed binding to the TESCO sequence and co-immunoprecipitation assays have demonstrated that FOXL2 and ERα physically interact (Uhlenhaut et al., 2009). Moreover, it has been shown in this thesis that mutation of all identified FOX and ERE sites in the TESCO sequence resulted in a loss of the synergistic repression effect mediated by ERα *in vitro*, though the repressive effect of FOXL2 was persisting. Analyses of the same mutations in the TESCO element *in vivo* showed a de-repression of TESCO:CFP in the adult ovary (Uhlenhaut et al., 2009). These data suggest that a potential FOXL2/ERα complex might bind to either FOX or ERE sites in the TESCO element and that *Sox9* repression is mediated via these sites in the TESCO element in the adult ovary. A possible explanation for the differing results *in vitro* and *in vivo* could be the presence of some minor interaction sites for FOXL2 in the mutated TESCO sequence. These might not play a crucial role *in vivo* and TESCO activity becomes de-repressed upon loss of the major FOX and ERE sites. However, in the *in vitro* co-transfection assays these minor sites might still mediate the repression of TESCO activity due to the excessive amount of available FOXL2. Although the conclusions which can be drawn from the *in vitro* assays should be regarded carefully due to the limitations of this artificial system, the results stress the same trend seen *in vivo*: the importance of the synergistic repressive effect of FOXL2 and ERα on TESCO activity

In contrast to the adult ovary, FOXL2 seems not to be the crucial factor for TESCO repression during embryogenesis as loss of *Foxl2* does not result in the

up-regulation of TESCO activity or *Sox9* expression before birth. In mice, no steroidogenesis takes place during XX embryonic development (Pannetier et al., 2006a), thus no production of estrogens occurs during this time period. It is possible that, due to the lack of estrogen signalling, ERα is not translocated into the nucleus and thus cannot interact with FOXL2 which results in the inability of FOXL2 to repress TESCO. To understand this mechanism better, it should be analysed via ChIP assays whether FOXL2 is able to bind to the TESCO element in the absence of ERα during embryogenesis. In contrast to mice, production of estrogens has been found during early ovarian development in the goat. It has been shown that FOXL2 activates *Cyp19a1* expression in goat (Pannetier et al., 2006a), indicating that loss of *Foxl2* expression in XX goats with female-to-male sex reversal (PIS mutation) might also result in the additional absence of estrogens during embryonic development. The phenotype in PIS mutant goats is evident much earlier than in *Foxl2* mutant mice, suggesting that indeed estrogens play an important role in repressing the testis pathway during embryonic development in the goat. Loss of *Foxl2* therefore seems to be the primary cause of the XX sex reversal in PIS animals.

In XX mice however, double mutations of both ERα and ERβ ($\alpha\beta$ERKO) result in mostly normal pre-pubertal ovaries (Couse et al., 1999). In such mice, the early differentiation of the reproductive tract occurs normally and all Müllerian duct-derived structures are developed. However, the pre-pubertal ovaries possess adult-like follicles indicating a precocious maturation of the ovary. In contrast, ovaries of adult $\alpha\beta$ERKO mice show the appearance of some seminiferous tubule like structures and Sertoli-like cells. Moreover, *Sox9* expression becomes

up-regulated, resulting in an adult sex-reversed phenotype similar to the one described in adult *Foxl2*$^{\Delta/\Delta}$ mice (Uhlenhaut et al., 2009), although less pronounced. While the phenotype of $\alpha\beta$ERKO mice first becomes evident after puberty, the phenotype of *Foxl2*$^{LacZ/LacZ}$ mice is visible as early as 7 dpp. These differences in timing indicate that FOXL2 cannot just depend on an interaction with ERs to regulate female sex determination.

7.3 FOXL2 interferes with SF1-mediated activation of TESCO *in vitro*

In this thesis it has been demonstrated that FOXL2 interacts directly with SF1. It was also shown that mutation of all putative FOX sites in the TESCO element did not result in a loss of TESCO repression mediated by FOXL2, suggesting that FOXL2 does not act, at least not exclusively, via these predicted binding sites. Moreover, the data suggests that FOXL2 can bind to DNA at the SF1 binding site and might be competing with SF1 for binding to the TESCO sequence. Recently a paper was published which postulated a new FOXL2-specific DNA binding site GTCAAGG(T/C)CA (Benayoun et al., 2008). This new binding site resembles very closely the SF1 binding site (T/C)(T/C)AAGG(T/C)C(G/A). Taken together, these data allows to hypothesise several ways by which FOXL2 could interact with SF1 to regulate TESCO activity (Figure 7.1): (i) FOXL2 (possibly together with ERα) might physically interact with SF1 and thereby reduce the amount of SF1 available to activate TESCO, (ii) FOXL2 (possibly together with ERα) might interact with DNA-bound SF1, thereby inhibiting its

transcriptional activation capability, (iii) FOXL2 might be competing with SF1 for binding to the TESCO element, resulting in a repression of TESCO rather than an activation. In future experiments, the exact binding sites for FOXL2 in the TESCO element should be analysed e.g. by *in vivo* footprinting assays, to determine if FOXL2 is indeed able to bind to SF1 binding sites or if it could possibly bind to other sites which differs from the consensus forkhead factor binding site. Further structural analyses should also determine the interaction between FOXL2/ERα and FOXL2/SF1 in more detail to find out which domains might be involved in protein-protein interactions and how they might bind to DNA as protein complexes.

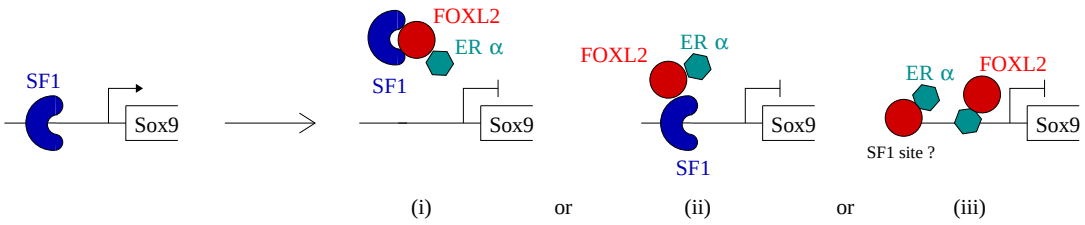


Figure 7.1: **Model of possible interactions between FOXL2 and SF1 to regulate *Sox9* expression in XX gonads.**
SF1 initiates low expression of *Sox9* in the XX gonad, but this is then down-regulated and it is maintained in a repressed state in the granulosa cells throughout life. It is likely that the repression is mediated via the TESCO element, and FOXL2 (and ERα) could contribute to this in the embryo and then become solely responsible after birth, in several ways: (i) FOXL2 (+ ERα?) could interact with SF1, preventing the latter from binding DNA, (ii) FOXL2 (+ ERα?) could interact with DNA-bound SF1, interfering with its ability to activate transcription or actively recruit co-repressors (iii) FOXL2 interacts with ERα and the complex might bind to TESCO at FOX sites, EREs or possibly at SF1 binding sites and directly mediate repression.

7.4 FOXL2 represses TESCO activity and endogenous *Sox9* expression postnatally

The *in vivo* analyses conducted in this thesis, showed that loss of *Foxl2* expression resulted in an up-regulation of TESCO activity in XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads. This indicates that FOXL2 is repressing TESCO activation *in vivo* as well as *in vitro*. However, the de-repression of TESCO activity was only seen postnatally and not at any of the time points analysed during embryogenesis. Endogenous *Sox9* expression was also up-regulated postnatally in the same cells as CFP, in cells surrounding oocytes. These cells are likely to have been granulosa cells as they also express β-galactosidase, a marker expressed under the control of the endogenous *Foxl2* promoter. Thus, it seems that the de-repression of both TESCO activity and endogenous *Sox9* expression occurs in granulosa cells which then take on Sertoli-like properties. In *Foxl2* mutant mice, these cells are organised in only one or two layers around the oocyte, in contrast to several layers of granulosa cells in wild-type ovaries. This seems to be due to a failure in proliferation (Uda et al., 2004) as might be expected if they are Sertoli cells. The up-regulation of *Sox9* expression in this layer of supporting cells indicates a switch from the female to a male fate. A similar result was obtained in the conditional *Foxl2*$^{\Delta/\Delta}$ mice where, upon loss of *Foxl2* in the adult ovary, both TESCO:CFP and *Sox9* became expressed in the layer of granulosa cells surrounding the oocytes (Uhlenhaut et al., 2009). Taken together, these results indicate that FOXL2 is indeed necessary to repress *Sox9* postnatally and that this regulation is accomplished via the TESCO element *in vivo*.

However, it is also possible that the loss of FOXL2 results in a de-repression of endogenous *Sox9* independent of the TESCO element. The de-repressed endogenous SOX9 could then act back on the TESCO element in a positive feedback loop in the same way that SOX9 contributes to its own regulation during early testis differentiation (Sekido and Lovell-Badge, 2008). It is thought that SOX9 levels need to reach a critical threshold first before such a feedback loop can be established. Thus, if loss of *Foxl2* indeed resulted in a de-repression of *Sox9* expression before activation of the TESCO element, there might be a detectable delay between the onset of endogenous *Sox9* expression and activation of CFP in XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ gonads. However, CFP activation preceded the de-repression of *Sox9* expression in such gonads. While a few CFP positive cells could already be found, endogenous *Sox9* expression was not detectable at P0, but it could be seen at 7 dpp. This is concordant with the finding that XX *Foxl2*$^{LacZ/LacZ}$ ovaries are indistinguishable from wild-type ovaries at birth but phenotypic differences were detected at 7 dpp (Schmidt et al., 2004; Uda et al., 2004). In future experiments, the timing and relationship between TESCO:CFP and SOX9 should be analysed in more detail and a closer look should be taken at the expression profile of the two proteins between P0 and 1 week of age. Also, the conditional *Foxl2* mutant mice (*Foxl2*$^{fl/fl}$) could be used to further analyse the de-repression of both TESCO activity and *Sox9* expression in the mutant ovaries. By crossing them with the TESCO:CFP reporter line it would be possible to analyse the exact timing between the loss of FOXL2 and the first appearance of CFP or SOX9. It would also be interesting to analyse the effect of loss of *Foxl2* on TESCO activity in the context of a *Sox9* null background, using the conditional

Sox9 null allele ($Sox9^{fl/fl}$) (Chaboissier et al., 2004). In this background, any de-repression of TESCO:CFP would have to be independent from endogenous SOX9 and instead directly due to the loss of FOXL2-mediated repression on the TESCO element. A different approach would be to first determine whether the TESCO element is the only regulatory element necessary to control *Sox9* expression in the gonad by generating a targeted deletion of the TESCO enhancer ($TESCO^{\Delta}$). The deletion of the TESCO element *in vivo* should result in a complete loss of gonadal *Sox9* expression. The TESCO:CFP reporter and the $Foxl2^{LacZ}$ allele could then be bred onto the $TESCO^{\Delta}$ background, where any de-repression of TESCO:CFP would be SOX9 independent.

Both TESCO and *Sox9* become robustly de-repressed with increasing age in the XX *TESCO:CFP;* $Foxl2^{LacZ/LacZ}$ gonads, suggesting that *Foxl2* becomes the only important gene to maintain *Sox9* repression in the adult. These findings were substantiated in the conditional $Foxl2^{\Delta/\Delta}$ mice, where deletion of *Foxl2* in 8 week old females resulted in the de-repression of *Sox9* and the transdifferentiation of granulosa cells into Sertoli cells (Uhlenhaut et al., 2009). Taken together these data indicate that FOXL2 is indeed a only critical factor needed to repress *Sox9* expression in the adult ovary. Interestingly, de-repression of *Sox9* starts from 1 week of age in XX *TESCO:CFP;* $Foxl2^{LacZ/LacZ}$ gonads, suggesting that FOXL2 becomes important for repression of *Sox9* expression at this time. In future experiments, *Foxl2* should be deleted at different time points around and after birth, using the conditional $Foxl2^{fl/fl}$ mice, to determine the exact time point from which *Foxl2* acts as the only critical factor required to repress *Sox9* expression postnatally. Moreover, using the conditional $Foxl2^{fl/fl}$ mice, it would be inter-

esting to analyse whether deletion of *Foxl2* just before birth (i.e. *Foxl2* would be expressed during most of the embryonic development) results in the same postnatal phenotype as seen in the $Foxl2^{LacZ/LacZ}$ mice (where *Foxl2* is never expressed). It is possible that loss of *Foxl2* from the early stages of embryogenesis results in the activation of redundant pathways to ensure proper ovarian development.

In future experiments, the effect of *Foxl2* misexpression in XY gonads should also be addressed. It has already been shown that misexpression of transgenic *Foxl2* under the control of an ubiquitous heat-shock inducible promoter in XY gonads resulted in disorganised tubules and ovotestis-like structures at 13.5 dpc (Ottolenghi et al., 2007). However, no further analyses have been carried out in the gonads of these mice. It would be interesting to analyse both endogenous *Sox9* expression and TESCO:CFP reporter expression upon misexpression of *Foxl2* in XY gonads. If FOXL2 is indeed repressing *Sox9* via TESCO, misexpressed *Foxl2* should result in the down-regulation of both TESCO:CFP and endogenous *Sox9* in these gonads. To test this hypothesis, a construct for conditional misexpression of *Foxl2* has already been designed (IZ/Foxl2) and injected into fertilised mouse eggs. Mice carrying this transgene will express the *LacZ* gene ubiquitously and, upon cre-mediated recombination, HA-tagged *Foxl2* will be expressed from a CMV promoter (Figure 7.2). These mice will be crossed to the TESCO:CFP line and to mice expressing a gonad-specific cre-recombinase (e.g. Sf1:Cre, Amh:Cre) to analyse the effect of *Foxl2* expression on both TESCO activity and *Sox9* expression in the XY gonad . Another approach could be to analyse the effect of *Foxl2* transfection into cultured Sertoli cells *in vitro*. Unfortunately, in the supposed Sertoli cell line TM4, SOX9 protein could not be

detected either by immunohistochemistry or by Western blot analysis (data not shown and Beverdam et al., 2003). Therefore these cell line could not be used to analyse the potential effect of FOXL2 on *Sox9* expression *in vitro*. In future experiments, primary cultures of Sertoli cells from the TESCO:CFP reporter mouse line could be employed to analyse the effect of misexpression of *Foxl2* on both TESCO activity and endogenous *Sox9* expression *in vitro*.

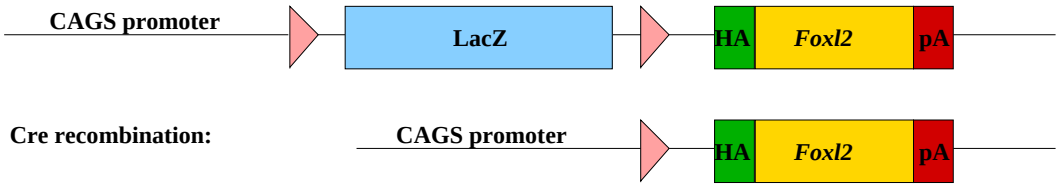


Figure 7.2: **IZ/Foxl2: A construct for conditional misexpression of *Foxl2*.** Mice carrying this transgene will express β-galactosidase ubiquitously. Upon cre-mediated recombination the two loxP sites (pink triangles) will recombine and *Foxl2*, which is N-terminally tagged with a HA-epitope, will be expressed.

7.5 *Sox9* expression is not up-regulated in oocyte-depleted ovaries

For a long time it had been proposed that oocytes are essential to maintain granulosa cell fate and that the loss of oocytes could cause a switch from granulosa cells to Sertoli cells (McLaren, 1991). In this thesis the question was addressed whether the loss of oocytes resulted in a postnatal up-regulation of *Sox9* expression comparable to the de-repression seen in XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ mice. When oocytes were specifically depleted by activation of a conditional form

of the diphtheria toxin fragment A (DTA) (Ivanova et al., 2005) using ZP3:Cre, no postnatal *Sox9* expression was detected in these ovaries at any of the postnatal time points analysed. Similar results were obtained using Gdf9:Cre as well as an inducible approach which allowed the deletion of oocytes in the adult ovary (Uhlenhaut et al., 2009). These results, compared to the analyses in the XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$ and XX *Foxl2*$^{\Delta/\Delta}$ mice, show that it is not the loss of oocytes, but the loss of a sole gene, *Foxl2*, which leads to up-regulation of *Sox9* expression in granulosa cells, resulting in their transdifferentiation to Sertoli cells. Interestingly, it has also been shown in neonatal rats, that depletion of oocytes by γ-irradiation resulted in a survival of granulosa cells which developed morphological characteristics of Sertoli cells, but did not up-regulate *Sox9* expression (Guigon et al., 2005). However, these experiments were not conclusive as not all oocytes were eliminated. In further experiments, the oocyte-depleted ovaries (ZP3:Cre, R26DTA) should be analysed in more detail regarding their morphological characteristics as well as further marker analyses to establish which cell types are still present upon loss of oocytes and how the granulosa cells are effected in terms of cellular and molecular properties. However, it remains possible that oocytes are required during late fetal stages to prevent a fate change from follicle to Sertoli cells. To analyse this hypothesis, oocytes have to be depleted during embryonic development, e.g. by disrupting the progress of meiosis.

7.6 WNT4 signalling partially represses TESCO activity during embryonic development

It has been shown that *Wnt4*, when expressed at high levels, or stabilised β-catenin can antagonise the FGF9/FGFR2 feedback loop (Kim et al., 2006b) and prevent the maintenance of *Sox9* expression (Figure 7.3). This suggests an antagonism between the two signalling pathways, such that FGF9 tips the balance towards testis development and WNT4 towards ovarian development. This thesis presents the first evidence that *Wnt4* is also involved in the repression of TESCO activity and *Sox8*/*Sox10* expression during embryonic development.

Homozygous loss of *Wnt4* resulted in de-repression of TESCO from 14.5 dpc onwards, suggesting that *Wnt4* is at least partially responsible for TESCO repression during embryonic development. However, although TESCO:CFP was de-repressed in those gonads, no endogenous *Sox9* could be detected, indicating that additional factors are still repressing *Sox9* during this time.

Moreover, as TESCO activity in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads becomes de-repressed only from 14.5 dpc, WNT4 cannot be the critical factor responsible for its repression prior to this point. It is possible that other *Wnts* which are expressed in the gonad compensate for the loss of *Wnt4*. However, other factors could also be involved in the early repression of TESCO activity (and *Sox9* expression). R-spondin1 is one possible candidate (Figure 7.3). RSPO1 was first identified to play a role in sex determination in human patients which display XX female-to-male sex reversal due to homozygous LoF mutations of *R-SPO1* (Parma et al., 2006). In the mouse, *Rspo1* is expressed in somatic cells of the

indifferent gonad in both sexes at 10.5 dpc and then becomes female-specific around 12.5 dpc (Parma et al., 2006). Mice with a targeted null mutation of *Rspo1* (Rspo1$^{-/-}$) show partial XX female-to-male sex reversal (Chassot et al., 2008; Tomizuka et al., 2008). *Rspo1* is still expressed in XX *Wnt4*$^{-/-}$ gonads (Chassot et al., 2008). Thus it is possible that *Rspo1* might be responsible for the repression of TESCO prior to 14.5 dpc in the XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads. In future experiments, the effect of loss of *Rspo1* expression on both TESCO activity and endogenous *Sox9* expression should be determined in XX gonads from 11.5 dpc onwards.

As homozygous *Wnt4* mutant mice die within 24 h after birth due to kidney failure, the gonadal phenotype of those mice could only be analysed until P0. Recently, a conditional *Wnt4* mutant mouse line has been described (*Wnt4*$^{fl/fl}$), which upon cre-mediated deletion during early embryogenesis, displays a phenotype consistent with the *Wnt4*$^{-/-}$ mice (Shan et al., 2009). It would be very interesting to analyse the effect of later loss of *Wnt4* expression on both TESCO activity and *Sox9* expression. If FOXL2 is indeed the only critical factor repressing TESCO activity after birth, postnatal loss of *Wnt4* should not result in a de-repression of either TESCO activity or *Sox9* expression.

Although CFP expression was detected in XX gonads of both *TESCO:CFP; Wnt4*$^{-/-}$ and *TESCO:CFP; Foxl2*$^{-/-}$ mice at P0, no de-repression of *Sox9* could be found in either single mutant. Interestingly, XX gonads of mice with double homozygous mutations in *Foxl2* and *Wnt4* not only show a stronger de-repression of TESCO activity at P0, but also up-regulation of endogenous *Sox9* expression.

Moreover, XX gonads of double mutant mice show a much more severe phenotype than the single mutants including the formation of seminiferous tubule-like structures. This suggests that both FOXL2 and WNT4 are necessary to repress *Sox9* expression in XX gonads around the time of birth. However, more detailed analyses of de-repression of both TESCO activity and endogenous *Sox9* during the time of embryonic development are necessary in the gonads of XX *TESCO:CFP; Foxl2*$^{LacZ/LacZ}$*; Wnt4*$^{-/-}$ mice to better understand the synergistic effect of the two genes.

7.7 *Sox8* and/or *Sox10* expression is de-repressed in embryonic *Wnt4* mutant XX gonads

The de-repression of TESCO in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads coincides with the activation of the expression of *SoxE* genes from 14.5 dpc until P0. The SOXE group of proteins comprises SOX8, SOX9 and SOX10, which share high sequence homology even outside the HMG box. Like *Sox9*, both *Sox8* and *Sox10* are known to be expressed in the XY gonad around the time of sex determination (Schepers et al., 2003; Polanco et al., 2010).

Studies in mice with double mutations of both *Sox8* and *Sox9* have suggested that the two genes might be involved in redundant pathways immediate after *Sox9* up-regulation and during subsequent testis development (Chaboissier et al., 2004; Barrionuevo et al., 2009). It has been shown that *Sox9* is essential for the initiation of testis differentiation as the homozygous deletion of *Sox9* prior or at the time of sex determination causes XY sex reversal (Chaboissier et al., 2004).

Moreover, conditional deletion of *Sox9* at 14.5 dpc, after the successful initiation of testis differentiation, results in normal embryonic and early postnatal testis development. These mice show a late sterility around 7 months of age due to a loss of spermatogonial stem cells and subsequent abrogation of spermatogenesis (Barrionuevo et al., 2009). On the other hand, homozygous loss of *Sox8* leads to an 80% sterility in male mice, while XY $Sox8^{+/-}$ and XX $Sox8^{-/-}$ mice are reproductively normal with no sex reversal. However, about 20% of XY $Sox8^{-/-}$ mice are able to produce a reduced number of offspring before they become sterile at 5 months of age (O'Bryan et al., 2008). Mice carrying a heterozygous conditional mutation of *Sox9* on a *Sox8* null background ($Sox8^{-/-}$, $Sox9^{\Delta/-}$) display a more severe phenotype than single $Sox8^{-/-}$ or $Sox9^{\Delta/-}$ mice. XY $Sox8^{-/-}$, $Sox9^{\Delta/-}$ gonads show abnormally shaped testis cords and defects in the formation of the coelomic vessel. Moreover, these gonads have a reduced number of seminiferous tubules and show partial sex reversal with areas resembling ovarian structures and lacking *Amh* expression at 15.5 dpc (Chaboissier et al., 2004). Homozygous double mutations of the two genes after 14.5 dpc using the *Sox9* conditional allele and Amh:Cre ($Sox8^{-/-}$, $Sox9^{\Delta/\Delta}$) result in progressive testis cord degradation with reduced numbers of testis cords containing a large amount of apoptotic cells. At 2 months of age, the testes of such mice completely lack any tubular structures and are mainly composed of Leydig cells resulting in a primary infertility. Moreover, some XY mice with a homozygous double mutation in both genes displayed a residual uterus in addition to testicular structures, the formation of which is attributed to an almost complete down-regulation of *Amh* expression (Barrionuevo et al., 2009). All this data indicates that *Sox8* and *Sox9* act redundantly during

testis differentiation as both are important for proper Sertoli cell specification and maintenance of *Amh* expression.

Although a targeted mutation of *Sox10* has been described (Britsch et al., 2001), the effect of loss of *Sox10* in the gonad has not yet been determined. Recently, it has been demonstrated that misexpression of *Sox10* in XX mice under the control of the *Wt1* regulatory region, causes XX female-to-male sex reversal at 13.5 dpc (Polanco et al., 2010).

Since SOX9 is still repressed in the XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads analysed in this thesis, the detected SOXE signal must be due to SOX8 and/or SOX10. In future experiments, it will be necessary to determine by *in situ* hybridisation which of the two genes, if not both, are expressed in the mutant gonads. The de-repression of the *SoxE* gene(s) in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads suggests that *Sox8* and/or *Sox10* might normally be repressed by WNT4 signalling in XX gonads during this time of embryonic development. It might therefore be possible, that the up-regulation of *Sox8* and/or *Sox10* expression could be involved in causing the masculinisation described in XX *Wnt4*$^{-/-}$ gonads (Vainio et al., 1999) in the absence of *Sox9*. Furthermore, it would be interesting to know whether *Sox8* misexpression can cause XX sex reversal similar to *Sox9* and *Sox10*.

Recently, it has been shown that both SOX8 and SOX10 in combination with SF1 can activate TESCO *in vitro*, although not as strongly as SF1 and SOX9 (Polanco et al., 2010). According to this data, it might also be possible that loss of *Wnt4* in XX gonads results in a de-repression of endogenous *Sox8* and/or *Sox10* which then in turn activates the TESCO:CFP reporter. To further analyse

this possibility, the *TESCO:CFP; Wnt4* mutant mice could be crossed to the *Sox8* null background, the *Sox8*/*Sox9* double null background and the *Sox10* null background. On the other hand, no gonad-specific regulatory elements for *Sox8* and *Sox10* have been described. As *Sox8* and/or *Sox10* de-repression temporally coincides with TESCO up-regulation in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads, it is reasonable to speculate that a gonadal *Sox8* and/or *Sox10* enhancer might be regulated similarly to the TESCO element.

7.8 Additional factors must be involved in repressing *Sox9* in embryonic XX gonads

While TESCO:CFP became de-repressed in XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads at 14.5 dpc, endogenous *Sox9* was still repressed. This may indicate that other factors are involved in repressing *Sox9* expression during embryogenesis (Figure 7.3), independently of the TESCO element, although it is also possible that the latter is integrated in a site more favourable for expression, e.g. next to an active gene. Both RSPO1 and WNT4 act by stabilising β-catenin (Komiya and Habas, 2008; Chassot et al., 2008). It has already been shown that expression of a stabilised form of β-catenin in XY gonads at 11.5 dpc led to male-to-female sex reversal (Maatouk et al., 2008). Both *Sox9* and *Amh* expression were dramatically reduced in the XY gonads of these mice after 12.5 dpc, suggesting that β-catenin is indeed able to antagonise *Sox9* expression in the gonad, although the mechanism is still unknown. In future experiments, it would be interesting to analyse the effect of gonadal loss of β-catenin on *Sox9* expression and TESCO ac-

tivity by crossing conditional β-catenin null mutant mice (Huelsken et al., 2001) to mice expressing a gonad-specific cre-recombinase (e.g. Sf1:Cre, Amh:Cre) and also to the TESCO:CFP reporter mice.

In future analyses it will be necessary to search for additional factors which are involved in the repression of *Sox9* in the XX gonad during embryogenesis. This search could be performed via different approaches, e.g. by comparing differences in gene expression between XX and XY gonads during different time points around sex determination, as done by Nef et al. (2005), Beverdam and Koopman (2006) and Bouma et al. (2007). Another possible approach would be forward genetic screening, such as ENU mutagenesis as performed by Bogani et al. (2009). Subsequently, mutations of identified candidate genes should be analysed *in vivo*. However, mutations in genes which are important in early embryonic development could result in embryonic lethality prior to the time of sex determination and therefore conditional approaches might be necessary. As an alternative to the classical expression-base screens, more global genomic approaches have recently been applied. In these studies, differences in gene expression profiles are mapped to chromosomal regions in the genome (eQTL). Recently, an eQTL analysis compared the gene expression levels in the developing gonad between a mouse strain sensitive to XY male-to-female sex reversal (C57BL/6J) versus a non-sensitive strain (129S1/SvImJ) at 11.5 dpc and found a number of novel genes and chromosomal loci which might be involved in the regulation of sex-related genes (Munger et al., 2009). This approach can be used to identify genetic network interactions on a global scale, as well as finding new regulators in sex determination, including both protein-coding and non-protein coding genes.

Non-protein coding genes, e.g. micro RNAs (miRNAs), are known to play important roles in the regulation of gene expression and it is possible that they account for the unknown factor regulating *Sox9* expression in the XX gonad. Micro RNAs are small non-coding RNAs of 21-23 nucleotides, which alter the expression of target genes by post-transcriptional inhibition or degradation of mRNA sequences to which they are complementary (for review see Bartel, 2004). Analyses in the adult subventricular zone of the central nervous system have shown that *Sox9* is a direct target of miR-124 (Cheng et al., 2009). It is possible that miR-124 might also be involved in the degradation of *Sox9* transcripts in the early XX gonad (Figure 7.3), resulting in the persistent repression of endogenous SOX9 in the XX *TESCO:CFP; Wnt4*$^{-/-}$ gonads while TESCO:CFP is de-repressed. Moreover, the brief burst of *Sox9* expression seen early in XX *Wnt4*$^{-/-}$ gonads (Kim et al., 2006b) could fail to establish the positive autoregulatory loop because miR-124 has become up-regulated and prevents further translation of SOX9. However, it is not known yet whether miR-124 is indeed expressed in the supporting cell precursors of the XX gonad at the right time to be involved in the repression of *Sox9*. Thus, in future experiments, the precise expression pattern of miR-124 in the gonad should be investigated at different time points during embryonic development. Furthermore, the effect of miR-124 misexpression on *Sox9* expression could be analysed in XY gonads or the effect of anti-miR (miRNA inhibitors) designed for miR-124 could be examined in XX gonads.

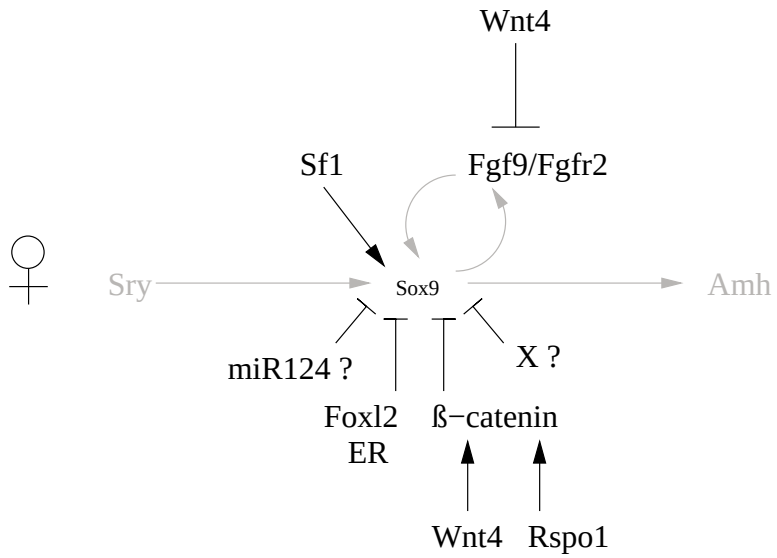


Figure 7.3: **Model of genetic interactions during ovarian development.** Possible genetic interactions in the XX gonad, including different mechanisms which could be involved in the down-regulation of *Sox9* in the female granulosa cell lineage.

7.9 Summary

In this thesis, it has been shown that the genes *Dax1*, *Sox4* and *Foxl2* (alone or in combination with ERα) have the potential to repress TESCO activity *in vitro*. It has also been demonstrated that the loss of *Foxl2* results in a postnatal de-repression of TESCO activity and endogenous *Sox9* expression in XX gonads, which is most evident in the adult. The de-repression of both TESCO and *Sox9* occurs in cells derived from granulosa cells, indicating a transdifferentiation of the female supporting cells to Sertoli cells. These data indicate that FOXL2 is the crucial factor to repress *Sox9* expression in the adult ovary and that this repression is mediated via the TESCO element (Figure 7.4). On the other hand, *Foxl2* is not critical for the repression of either TESCO or *Sox9* during embryonic development. Moreover, it has been shown in this thesis that WNT4 signalling could be partially responsible for *Sox9* repression during embryonic development from 14.5 dpc onwards via the TESCO element. As *Sox9* is still repressed in XX *Wnt4* mutant gonads, additional factors (e.g. *Rspo1* or miR-124) must be involved in its initial down-regulation at 11.5 dpc and persisting repression during embryonic development. Interestingly, both FOXL2 and WNT4 seem to play a role in the repression of *Sox9* around the time of birth, possibly via the TESCO element (Figure 7.4).

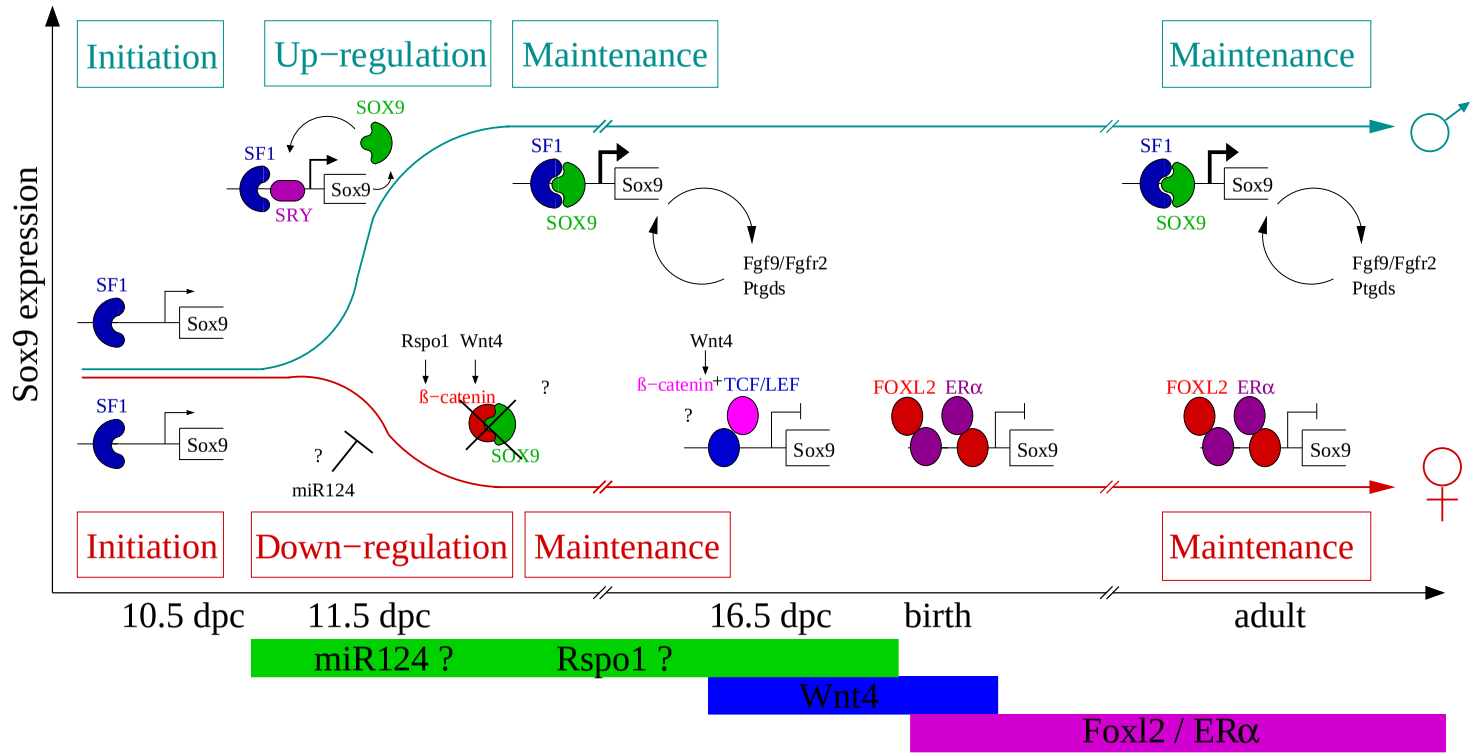


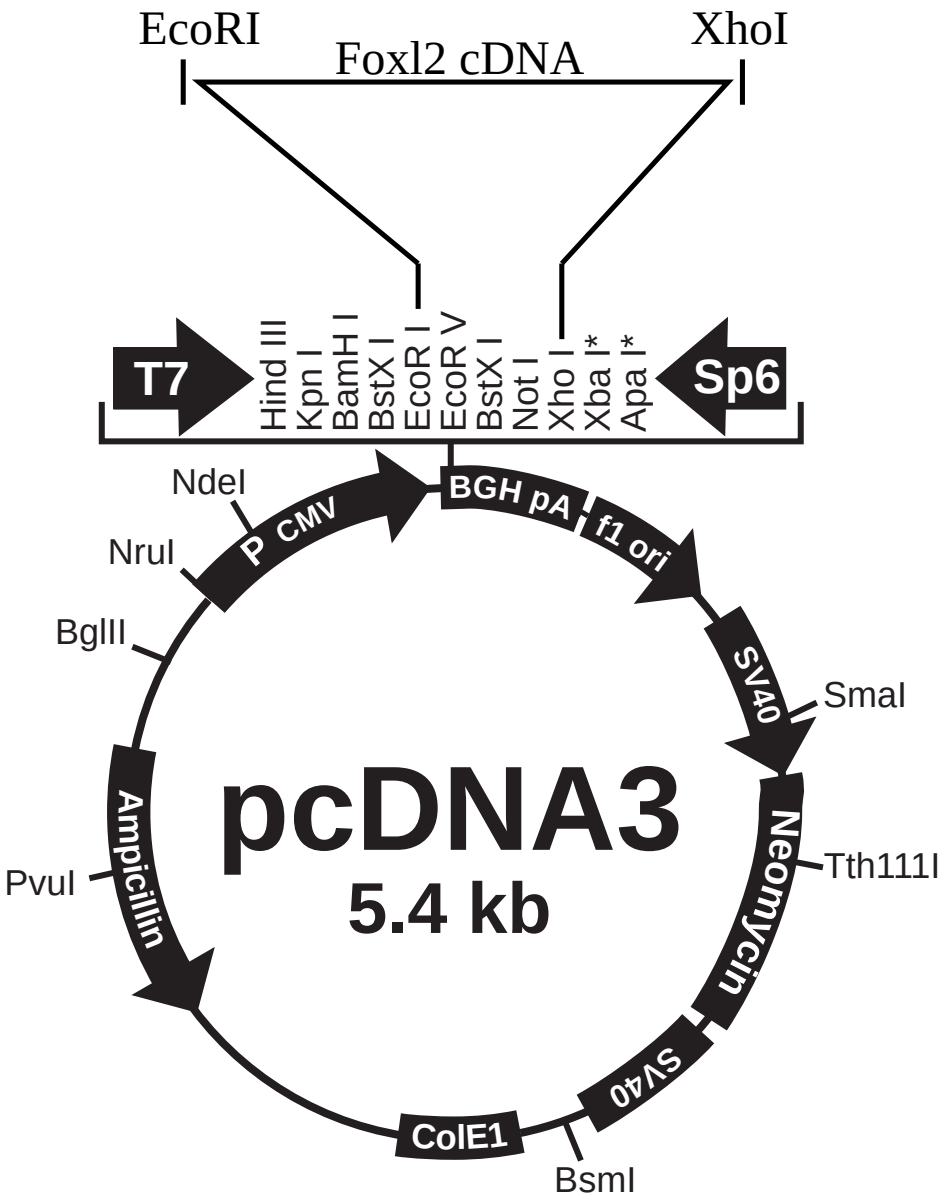
Figure 7.4: **Model of the regulation of gonadal *Sox9* expression.**
In the XY gonad, SF1 binds to the TESCO enhancer of *Sox9* and initiates a low *Sox9* expression, then SRY binds to TESCO and up-regulates *Sox9* expression, which is maintained by SOX9 itself and other factors such as FGF9 and PGD_2 signalling. In the XX gonad, the initial expression of *Sox9* is down-regulated at 11.5 dpc and the repression is maintained, both of which could possibly involve the TESCO element. It is not known yet which genes are responsible for this early down-regulation, though both miR-124 and Rspo1 might be good candidates. WNT4 signalling seems to be partially responsible for repression of TESCO activity from 14.5 dpc onwards. Both WNT4 and FOXL2 are involved in the repression of TESCO activity and *Sox9* expression at the time of birth. In the adult ovary, FOXL2 (in cooperation with ERα) is the crucial factor to repress *Sox9*, possibly via TESCO.

Chapter 8

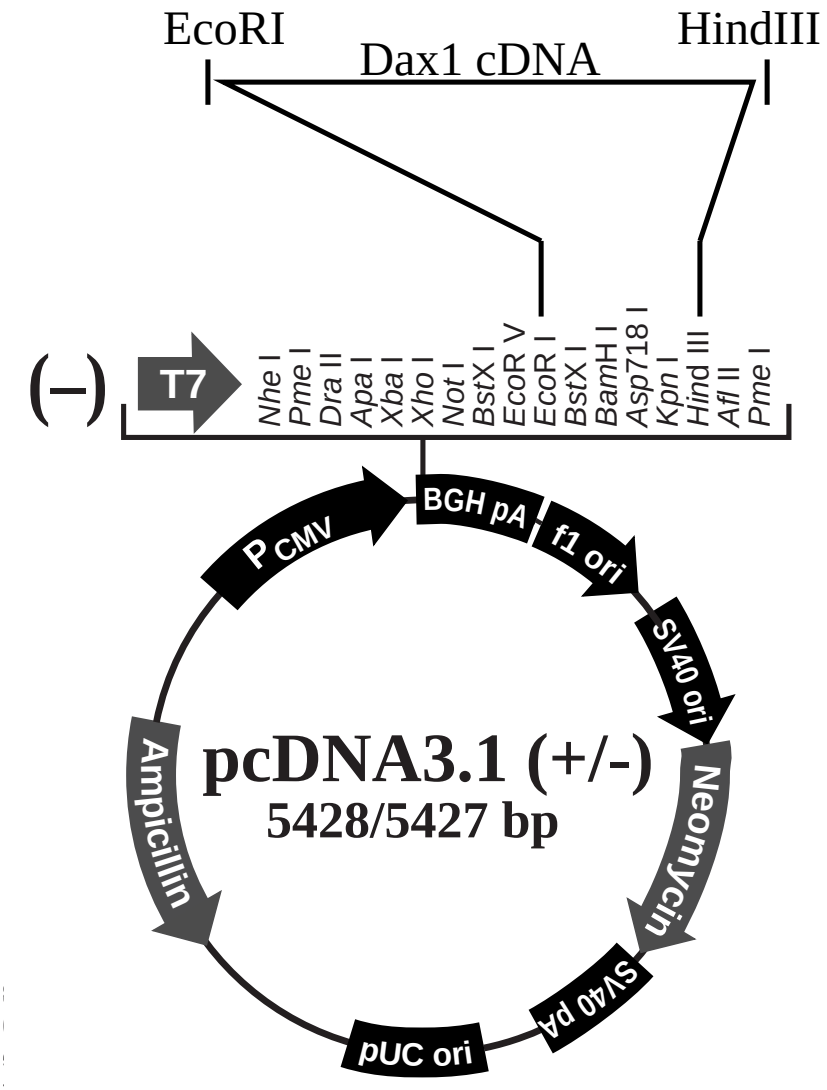
Appendices

8.1 Plasmid maps

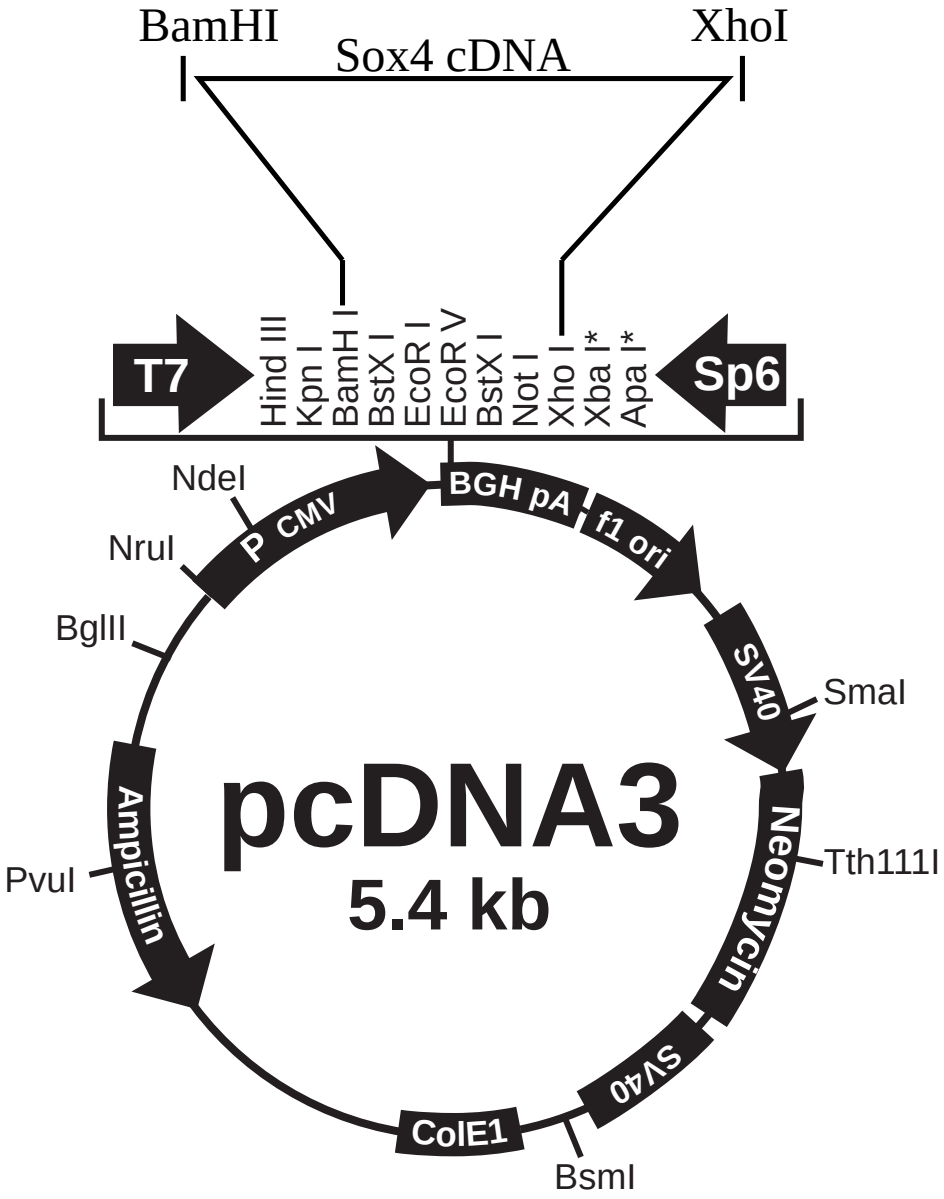
8.1.1 pcDNA-Foxl2



8.1.2 pcDNA-Dax1



8.1.3 pcDNA-Sox4



8.2 PCR-protocols

8.2.1 CFP PCR

Primer

Forward: 5'-GACCCTGAAGTTCATCTGCAC-3'

Reverse: 5'-GTGGCTGATGTAGTTGTACTC-3'

Size: 300 bp

PCR Protocol

H_2O	19.0 μl
10X buffer	2.5 μl
$MgCl_2$ [25mM]	1.25 μl
DMSO	1.0 μl
dNTP [25mM]	0.2 μl
primer for [50mM]	0.125μl
primer rev [50mM]	0.125μl
Taq DNA polymerase	0.125μl
DNA	0.5 μl

PCR Program

94°C	3 min	
94°C	45 sec	} 30x
53°C	45 sec	
72°C	1 min	
72°C	7 min	

8.2.2 Foxl2 PCR

Primer

FWB3: 5'-CAGATGATGGCCAGCTACCCCGAGC-3'

FWB4: 5'-GTTGTGGCGGATGCTATTCTGCCAGCC-3'

FWB5: 5'-GTAGATGGGCGCATCGTAACCGTGC-3'

Size: wild-type = 200 bp, mutant = 500 bp

PCR Protocol

H_2O	16.45μl
10X buffer	2.5 μl
$MgCl_2$ [25mM]	2.0 μl
DMSO	1.25 μl
dNTP [25mM]	0.2 μl
primer FWB3 [10mM]	0.5 μl
primer FWB4 [10mM]	0.5 μl
primer FWB5 [10mM]	0.5 μl
Taq DNA polymerase	0.1 μl
DNA	1.0 μl

PCR Program

94°C	3 min	
94°C	30 sec	} 35x
58°C	30 sec	
72°C	45 sec	
72°C	5 min	

8.2.3 Wnt4 PCR

Primer

Wnt4-E3for:	5'-CTTCACAACAACGAGGCTGGCAGG-3'
Wnt4-E4rev:	5'-CACCCGCATGTGTGTCAAGATGG-3'
Wnt4-neo-rev:	5'-GCATTGTCTGAGTAGGTGTCATTC-3'

Size: wild-type = 700 bp, mutant = 400 bp

PCR Protocol

H_2O	17.05μl
10X CAT-buffer	2.5 μl
dNTP [25mM]	0.25 μl
primer E3for [20mM]	1.0 μl
primer E4rev [20mM]	1.0 μl
primer neo-rev [20mM]	1.0 μl
Taq DNA polymerase	0.2 μl
DNA	2.0 μl

PCR Program

94°C	4 min	
94°C	1 min	} 45x
65°C	30 sec	} 45x
72°C	30 sec	} 45x
72°C	5 min	

8.2.4 Cre PCR

Primer

Forward: 5'-GGCGGATCCGAAAAGAAAA-3'

Reverse: 5'-CAGGGCGCGAGTTAGTAGC-3'

Size: 400 bp

PCR Protocol

H_2O	17.62μl
10X buffer	2.5 μl
$MgCl_2$ [25mM]	1.5 μl
DMSO	1.25 μl
dNTP [25mM]	0.2 μl
primer for [50mM]	0.31 μl
primer rev [50mM]	0.31 μl
Taq DNA polymerase	0.31 μl
DNA	1.0 μl

PCR Program

94°C	3 min	
94°C	45 sec	} 35x
54°C	45 sec	
72°C	45 sec	
72°C	7 min	

8.3 TESCO sequence alignment

CLUSTAL 2.0.12 multiple sequence alignment

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	TESCO_human	1389	2	TESCO_dog	1502	78
1	TESCO_human	1389	3	TESCO_mouse	1293	62
1	TESCO_human	1389	4	TESCO_rat	1378	60
2	TESCO_dog	1502	3	TESCO_mouse	1293	42
2	TESCO_dog	1502	4	TESCO_rat	1378	43
3	TESCO_mouse	1293	4	TESCO_rat	1378	86

Result:

```
TESCO_mouse      CATATGTCACATACCTAAGGTGAAAATATACGAGCCCTGTCTAAATCGGAACTCCAACCA 60
TESCO_rat        CATATGTCCCATACCTAAGGTGTAAATATACTAGCCTTGTG------GGAACTCCAACTA 54
TESCO_human      ----------ACATCACTGATGTAAATATACTAGACCTGTCTAAATCTAAACTCCAACTA 50
TESCO_dog        -------CAGACATCATGGATGTAAATATACTAGACCTGTCGAAATCTGAACTCCAAGTA 53
                           * * *   * ** ******** ** * ***         ********  *

TESCO_mouse      TGTACCATTTTC-CTTA------------AGGCCCCACAGGAAGAAAAAGGGAAAAAGAG 107
TESCO_rat        CGTACCATTTTTTCTTA------------AGCCCCCACAGGAAGAAATGGGGAAAATGAA 102
TESCO_human      CACACGAATTTTTTTA-------------ACGCTCCACAAAAAAGAAAATGAGAAAAGCA 97
TESCO_dog        TGCATCCTTTTTTTTTTTTTTTTTTTTCAGGCTCCACCAAAAGAAAAAGAGGAAAAGGA 113
                    *    ***   *             *  * ****   **  **      *** *

TESCO_mouse      AG------------------AGAGACCGA--------CTCTATTGTTGAATTACTGTTAG 141
TESCO_rat        AGCCTGGA--ACCCCGTATAGGAGTCAGGTAGCCTTTCTCCCTTGTCAACTAACTATTAG 160
TESCO_human      AAACTCAACAACACCCTAAAGGAGTCAGATCCTCTTTCTATATTGTCAGGTAATTGTTAG 157
TESCO_dog        AAACTCAACAAGCCCCTAAAGGAGTCAGATCCCCCT-CTATATTGTCAGGTAATTGTTAG 172
                 *                    *** * *         **   ****    * * * ****

TESCO_mouse      CAGAACTCAGCTGTAATACAGAACCATTTGAAAGGAATGCCAATTGAGTTCTGCCCAGCC 201
TESCO_rat        CAGAAATTGGCTGTAATACAGAGGCACTTTAAAGGAATGTCAATTGAGTTCTGCCCAGCC 220
TESCO_human      CAGAAATCAGCTGTAATAACAAGCCATTTTAAAGGAATGCCAATTCCATTCTATCCTGCT 217
TESCO_dog        CAGAAATGGGCTGTAATAACAAGCCATTTTAAAGGAATGTCAATTCAATTCTGTCTTGCT 232
                 ***** *  *********   *  ** ** ********* *****   ****  *  **

TESCO_mouse      TGAAGAAGACCCAGCCTC-GGCCTTTGTTCCTAACCTGGGCGG-TTTTCACAAAATAACA 259
TESCO_rat        TGAAGAAGACCCAGCCTCTGGCTTTTGTTCCTAACCTGGGCGGGTTTTCACAAAATAACA 280
TESCO_human      TAAAGAAGATCCAGAATC-TGCCTTTGTTCCTAACCTGGGCAG-TATGGAGAAAATAACA 275
TESCO_dog        TAAAGAAGATCCAGAGTC-TGCCTTTGTTCCTAACCTGAGCAG-TTTTGAGGAAATA--- 287
                 * ******* ****  **  ** *************** ** * * *  *  *****

TESCO_mouse      ATGCCTTCTTTCAGAAACTTTAGG-GCTAAGAAAGAGAAGACTCCA-CTCTCGCAGATAA 317
TESCO_rat        ATACCTTCTTTCAGAACCTTTGGG-ACTAAGAAAGGTACAAATCCT-CTCTCCCAGATAA 338
TESCO_human      ATACCTTCTTTCAGAAACTGTGGGGAATCTGAAAGGTAGGATTCCTGCTCTCCCAGATAA 335
TESCO_dog        ATACCTTCTTTCAGAAACTGTGGGGAATTTGCAAGGTAGGGCTCCTGCTTTCCCAGATAA 347
                 ** ************* ** * **   *  * ***  *    ***  ** ** *******

TESCO_mouse      GGGCTGGCAGAAGAGGTGGCAGATACCAACTACAGGGAGGTGGC-----TGCAGGAGTT- 371
TESCO_rat        GGGCTGGCAGAAGAGAAGGCAGCTACCAACTACAGGAAGGTAGC-----TACAGAAGTT- 392
TESCO_human      GAGCTGGCAGGAGAGGTGGCAGCTGTCAA--GGGGGAGGCTTGTGTGAGCCCTGGGGTCA 393
TESCO_dog        GCACGGGCAGGAGTGGGGACTGCTGGCCA--GGAGGAGGCTAGC-----TCTGGGGGTCA 400
                 *  * ***** ** *  * * * *  * *     **  * * *          *  **

TESCO_mouse      -----------CCCAGGGTCA-----AACACAAGTGCCTGGCTTCTTGGTGAGAGG--AA 413
TESCO_rat        -----------CCCGGGGCCA-----ACCACAAGTGCCTGGCTTCTTGGTGACAGG--CA 434
TESCO_human      GCCAGGAGCACCCTGGAGAGAAGACCAGGACCATCAGAAGGGAGCAAAGTAAGAGGTGCT 453
TESCO_dog        GCCAGCA----CCCAGGGAGAGGGCCAAC-CCAGCCACAGGGAGCGAGGTGAGGGG-GCG 454
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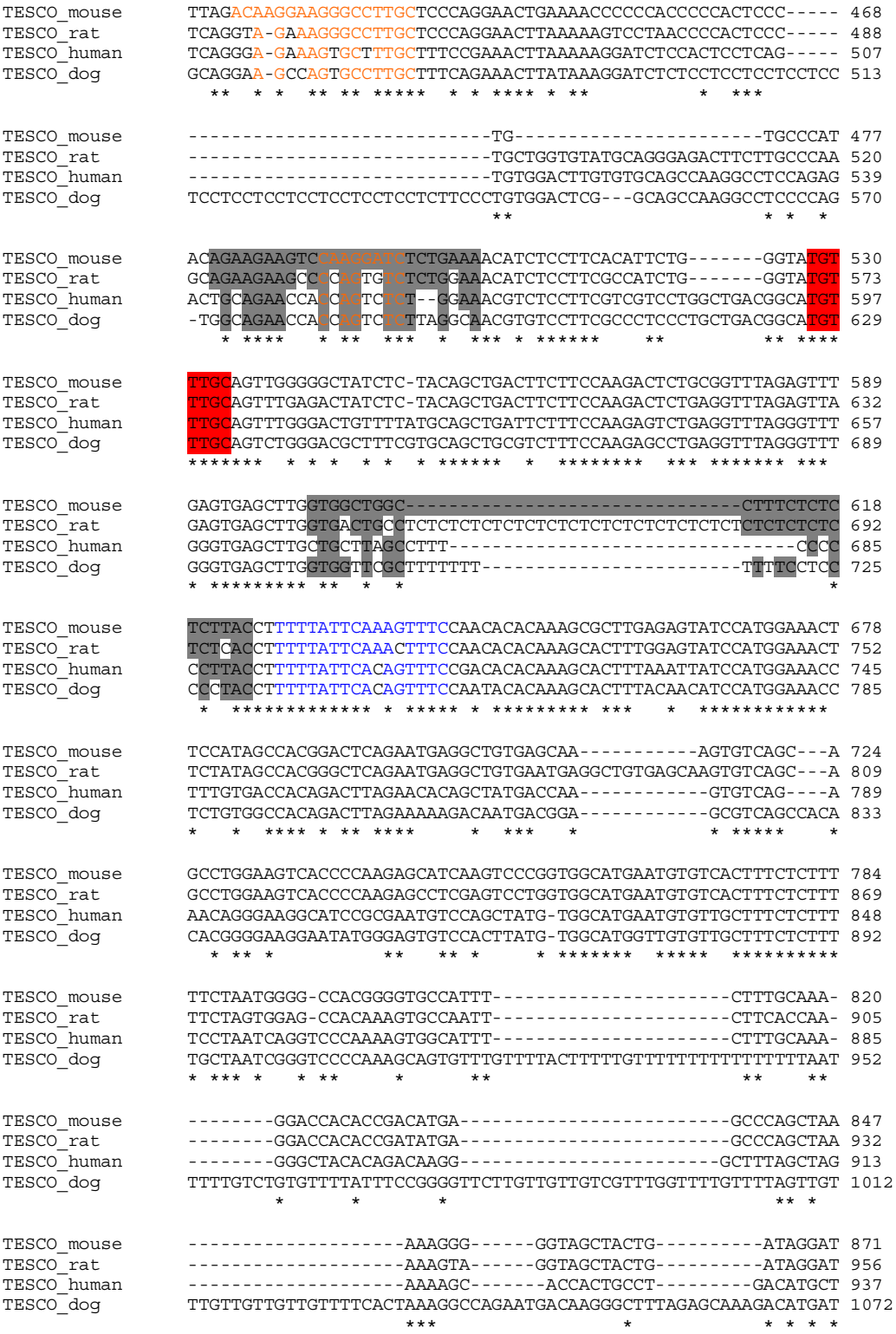
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TESCO_dog GCAGGAA-GCCAGTGCCTTGCTTTCAGAAACTTATAAAGGATCTCTCCTCCTCCTCCTCC 513
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TESCO_human ----------TGTGGACTTGTGTGCAGCCAAGGCCTCCAGAG 539
TESCO_dog TCCTCCTCCTCCTCCTCCTCTTCCCTGTGGACTCG---GCAGCCAAGGCCTCCCCAG 570
TESCO_mouse ACAGAAGAAGTCCAAGGATCTCTGAAAACATCTCCTTCACATTCTG-------GGTATGT 530
TESCO_rat GCAGAAGAAGCCCCAGTGTCTCTGGAAACATCTCCTTCGCCATCTG-------GGTATGT 573
TESCO_human ACTGCAGAACCACCAGTCTCT--GGAAACGTCTCCTTCGTCGTCCTGGCTGACGGCATGT 597
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TESCO_mouse TTGCAGTTGGGGGCTATCTC-TACAGCTGACTTCTTCCAAGACTCTGCGGTTTAGAGTTT 589
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TESCO_human TTGCAGTTTGGGACTGTTTTATGCAGCTGATTCTTTCCAAGAGTCTGAGGTTTAGGGTTT 657
TESCO_dog TTGCAGTCTGGGACGCTTTCGTGCAGCTGCGTCTTTCCAAGAGCCTGAGGTTTAGGGTTT 689
TESCO_mouse GAGTGAGCTTGGTGGCTGGC----------CTTTCTCTC 618
TESCO_rat GAGTGAGCTTGGTGACTGCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC 692
TESCO_human GGGTGAGCTTGCTGCTTAGCCTTT----------CCCC 685
TESCO_dog GGGTGAGCTTGGTGGTTCGCTTTTTTT----------TTTTCCTCC 725
TESCO_mouse TCTTACCTTTTTATTCAAAGTTTCCAACACACAAAGCGCTTGAGAGTATCCATGGAAACT 678
TESCO_rat TCTCACCTTTTTATTCAAACTTTCCAACACACAAAGCACTTTGGAGTATCCATGGAAACT 752
TESCO_human CCTTACCTTTTTATTCACAGTTTCCGACACACAAAGCACTTTAAATTATCCATGGAAACC 745
TESCO_dog CCCTACCTTTTTATTCACAGTTTCCAATACACAAAGCACTTTACAACATCCATGGAAACC 785
TESCO_mouse TCCATAGCCACGGACTCAGAATGAGGCTGTGAGCAA----------AGTGTCAGC---A 724
TESCO_rat TCTATAGCCACGGGCTCAGAATGAGGCTGTGAATGAGGCTGTGAGCAAGTGTCAGC---A 809
TESCO_human TTTGTGACCACAGACTTAGAACACAGCTATGACCAA----------GTGTCAG----A 789
TESCO_dog TCTGTGGCCACAGACTTAGAAAAAGACAATGACGGA----------GCGTCAGCCACA 833
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TESCO_human AACAGGGAAGGCATCCGCGAATGTCCAGCTATG-TGGCATGAATGTGTTGCTTTCTCTTT 848
TESCO_dog CACGGGGAAGGAATATGGGAGTGTCCACTTATG-TGGCATGGTTGTGTTGCTTTCTCTTT 892
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TESCO_dog TGCTAATCGGGTCCCCAAAGCAGTGTTTGTTTTACTTTTTGTTTTTTTTTTTTTTTAAT 952
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TESCO_rat --------GGACCACACCGATATGA----------GCCCAGCTAA 932
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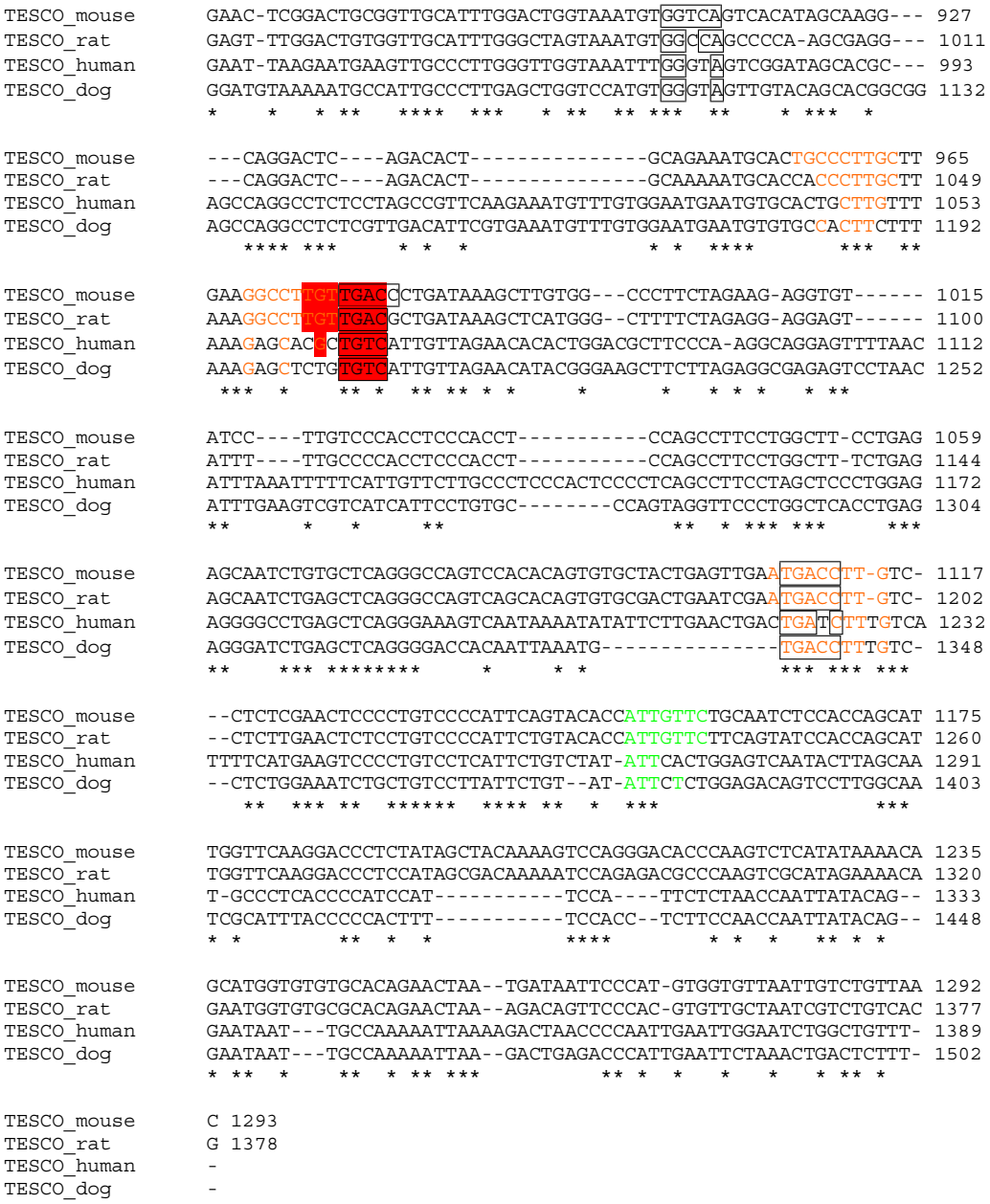
TESCO_mouse GAAC-TCGGACTGCGGTTGCATTTGGACTGGTAAATGTGGTCAGTCACATAGCAAGG--- 927
TESCO_rat GAGT-TTGGACTGTGGTTGCATTTGGGCTAGTAAATGTGGCCAGCCCCA-AGCGAGG--- 1011
TESCO_human GAAT-TAAGAATGAAGTTGCCCTTGGGTTGGTAAATTTGGGTAGTCGGATAGCACGC--- 993
TESCO_dog GGATGTAAAAATGCCATTGCCCTTGAGCTGGTCCATGTGGGTAGTTGTACAGCACGGCGG 1132
TESCO_mouse ---CAGGACTC----AGACACT---------------GCAGAAATGCACTGCCCTTGCTT 965
TESCO_rat ---CAGGACTC----AGACACT---------------GCAAAAATGCACCACCCTTGCTT 1049
TESCO_human AGCCAGGCCTCTCCTAGCCGTTCAAGAAATGTTTGTGGAATGAATGTGCACTGCTTGTTT 1053
TESCO_dog AGCCAGGCCTCTCGTTGACATTCGTGAAATGTTTGTGGAATGAATGTGTGCCACTTCTTT 1192
TESCO_mouse GAAGGCCTTGTTGACCCTGATAAAGCTTGTGG---CCCTTCTAGAAG-AGGTGT------ 1015
TESCO_rat AAAGGCCTTGTTGACGCTGATAAAGCTCATGGG--CTTTTCTAGAGG-AGGAGT------ 1100
TESCO_human AAAGAGCACCCTGTCATTGTTAGAACACACTGGACGCTTCCCA-AGGCAGGAGTTTTAAC 1112
TESCO_dog AAAGAGCTCTGTGTCATTGTTAGAACATACGGGAAGCTTCTTAGAGGCGAGAGTCCTAAC 1252
TESCO_mouse ATCC----TTGTCCCACCTCCCACCT-----------CCAGCCTTCCTGGCTT-CCTGAG 1059
TESCO_rat ATTT----TTGCCCCACCTCCCACCT-----------CCAGCCTTCCTGGCTT-TCTGAG 1144
TESCO_human ATTTAAATTTTTCATTGTTCTTGCCCTCCCACTCCCCTCAGCCTTCCTAGCTCCCTGGAG 1172
TESCO_dog ATTTGAAGTCGTCATCATTCCTGTGC--------CCAGTAGGTTCCCTGGCTCACCTGAG 1304
TESCO_mouse AGCAATCTGTGCTCAGGGCCAGTCCACACAGTGTGCTACTGAGTTGAATGACCTT-GTC- 1117
TESCO_rat AGCAATCTGAGCTCAGGGCCAGTCAGCACAGTGTGCGACTGAATCGAATGACCTT-GTC- 1202
TESCO_human AGGGGCCTGAGCTCAGGGAAAGTCAATAAAATATATTCTTGAACTGACTGATCTTTGTCA 1232
TESCO_dog AGGGATCTGAGCTCAGGGGACCACAATTAAATG---------------TGACCTTTGTC- 1348
TESCO_mouse --CTCTCGAACTCCCCTGTCCCCATTCAGTACACCATTGTTCTGCAATCTCCACCAGCAT 1175
TESCO_rat --CTCTTGAACTCTCCTGTCCCCATTCTGTACACCATTGTTCTTCAGTATCCACCAGCAT 1260
TESCO_human TTTTCATGAAGTCCCCTGTCCTCATTCTGTCTAT-ATTCACTGGAGTCAATACTTAGCAA 1291
TESCO_dog --CTCTGGAAATCTGCTGTCCTTATTCTGT--AT-ATTCTCTGGAGACAGTCCTTGGCAA 1403
TESCO_mouse TGGTTCAAGGACCCTCTATAGCTACAAAAGTCCAGGGACACCCAAGTCTCATATAAAACA 1235
TESCO_rat TGGTTCAAGGACCCTCCATAGCGACAAAAATCCAGAGACGCCCAAGTCGCATAGAAAACA 1320
TESCO_human T-GCCCTCACCCCATCCAT-----------TCCA----TTCTCTAACCAATTATACAG-- 1333
TESCO_dog TCGCATTTACCCCCACTTT-----------TCCACC--TCTTCCAACCAATTATACAG-- 1448
TESCO_mouse GCATGGTGTGTGCACAGAACTAA--TGATAATTCCCAT-GTGGTGTTAATTGTCTGTTAA 1292
TESCO_rat GAATGGTGTGCGCACAGAACTAA--AGACAGTTCCCAC-GTGTTGCTAATCGTCTGTCAC 1377
TESCO_human GAATAAT---TGCCAAAAATTAAAAGACTAACCCCAATTGAATTGGAATCTGGCTGTTT- 1389
TESCO_dog GAATAAT---TGCCAAAAATTAA--GACTGAGACCCATTGAATTCTAAACTGACTCTTT- 1502
TESCO_mouse C 1293
TESCO_rat G 1378
TESCO_human -
TESCO_dog -

8.4 TESCO deletion constructs

TESCO deletion constructs

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   1 CATATGTCAC ATACCTAAGG TGAAAATATA CGAGCCCTGT CTAAATCGGA ACTCCAACCA
  61 TGTACCATTT TCCTTAAGGC CCCACAGGAA GAAAAAGGGA AAAAGAGAGA GAGACCGACT  ΔC4
 121 CTATTGTTGA ATTACTGTTA GCAGAACTCA GCTGTAATAC AGAACCATTT GAAAGGAATG
 181 CCAATTGAGT TCTGCCCAGC CTGAAGAAGA CCCAGCCTCG GCCTTTGTTC CTAACCTGGG
 241 CGGTTTTCAC AAAATAACAA TGCCTTCTTT CAGAAACTTT AGGGCTAAGA AAGAGAAGAC
 301 TCCACTCTCG CAGATAAGGG CTGGCAGAAG AGGTGGCAGA TACCAACTAC AGGGAGGTGG  ΔC5
 361 CTGCAGGAGT TCCCAGGGTC AAACACAAGT GCCTGGCTTC TTGGTGAGAG GAATTAGACA
 421 AGGAAGGGCC TTGCTCCCAG GAACTGAAAA CCCCCCACCC CCACTCCCTG TGCCCATACA
 481 GAAGAAGTCC AAGGATCTCT GAAAACATCT CCTTCACATT CTGGGTATGT TTGCAGTTGG
 541 GGGCTATCTC TACAGCTGAC TTCTTCCAAG ACTCTGCGGT TTAGAGTTTG AGTGAGCTTG  ΔC6
 601 GTGGCTGGCC TTTCTCTCTC TTACCTTTTT ATTCAAAGTT TCCAACACAC AAAGCGCTTG
 661 AGAGTATCCA TGGAAACTTC CATAGCCACG GACTCAGAAT GAGGCTGTGA GCAAAGTGTC
 721 AGCAGCCTGG AAGTCACCCC AAGAGCATCA AGTCCCGGTG GCATGAATGT GTCACTTTCT
 781 CTTTTTCTAA TGGGGCCACG GGGTGCCATT TCTTTGCAAA GGACCACACC GACATGAGCC
 841 CAGCTAAAAA GGGGGTAGCT ACTGATAGGA TGAACTCGGA CTGCGGTTGC ATTTGGACTG  ΔC7
 901 GTAAATGTGG TCAGTCACAT AGCAAGGCAG GACTCAGACA CTGCAGAAAT GCACTGCCCT
 961 TGCTTGAAGG CCTTGTTGAC CCTGATAAAG CTTGTGGCCC TTCTAGAAGA GGTGTATCCT
1021 TGTCCCACCT CCCACCTCCA GCCTTCCTGG CTTCCTGAGA GCAATCTGTG CTCAGGGCCA  1.0kb
1081 GTCCACACAG TGTGCTACTG AGTTGAATGA CCTTGTCCTC TCGAACTCCC CTGTCCCCAT
1141 TCAGTACACC ATTGTTCTGC AATCTCCACC AGCATTGGTT CAAGGACCCT CTATAGCTAC
1201 AAAAGTCCAG GGACACCCAA GTCTCATATA AAACAGCATG GTGTGTGCAC AGAACTAATG
1261 ATAATTCCCA TGTGGTGTTA ATTGTCTGTT AAC
```

XXX	SF1 binding sites	T/C A A G G T/C C G/A
XXX	SOX binding sites	A G A A C A A T G G
XXX	FOXL2 core binding site	G/A T/C C/A **A A** C/T A

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