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Review

Molecular mechanisms in male determination and germ cell differentiation

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Abstract. Sex determination and gametogenesis are key processes in human reproduction, and any defect can lead to infertility. We describe here the molecular mechanisms of male sex determination and testis formation; defects in sex determination lead to a female phenotype despite the presence of a Y chromosome, more rarely to a male phenotype with XX chromosomes, or to intersex phenotypes. Interestingly, these phenotypes are often associated with other developmental malformations. In testis, spermatozoa are produced from renewable stem cells in a complex differentiation process called spermatogenesis. Gene expression during spermatogenesis differs to a surprising degree from gene expression in somatic cells, and we discuss here mechanistic differences and their effect on the differentiation process and male fertility.

Key words. Chromatin; gene expression; male infertility; sex determination; spermatogenesis.

Introduction

Sexual reproduction produces significant genetic variability in individuals. For sexual reproduction to occur, haploid gametes must form, and then fuse to form a zygote. Moreover, in mammals, the formation of germinal cells occurs in specialized organs, the gonads, which differ markedly between males and females, and whose development represents a large part of the process of sex determination. Both the formation of the gametes (gametogenesis) and of the gonads themselves are elaborate processes, which are still incompletely understood. Failures during both sex determination and gametogenesis are not lethal to the individuals, but are catastrophic for their genome since they makes its propagation impossible.
In this review we will describe both the molecular events of sex determination and their failure, and the role of chromatin and transcription factors in gametogenesis.

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Male reproductive development

At the time of fertilization, the sex of the future individual is determined by the genomic content of the gametes. The primary event in gonad development is the formation of the gonadal primordium, called the genital ridge. This structure is at first identical in both males and females, and can develop into testes or ovaries following specific stimuli. The information for the commitment of the genital ridge to testis formation resides in the Y chromosome. The discovery in the early 1990s of the sex-determining factor, called SRY for sex-determining region of the Y chromosome, was the first crucial step toward a general understanding of sex determination. Since then, several other genes have been found to have important roles: these either encode transcription factors such as SOX9, WT1, SF1, DAX1, and GATA4, or proteins involved in cell-to-cell signaling, such as MIS, WNT4, and FGF9.
Genetic analysis of intersex patients was crucial for understanding the molecular mechanisms involved in sex determination. Defects that affect male determination

result in malformations ranging from sexual ambiguity to complete sex reversal. Mutations in SRY correlate exclusively with gonadal dysgenesis [1], while mutations in SOX9 cause not only gonadal dysgenesis but also abnormal development of other organs, such as bone or adrenals, consistent with the expression profile of the disrupted gene [2]. Deletion of the gene for the signaling molecule WNT4 determines the masculinization of XX mice, while its duplication and overexpression in human males leads to feminization and sex reversal [3]. Mammalian sex determination is dosage sensitive at multiple steps in the genetic cascade that controls it: duplications of loci containing *SOX9* and *DAX1* also cause sex reversal [4, 5].

The following sections analyze the known role of the gene products mentioned above. These are summarized in table 1.

SRY

The information responsible for the development of male gonads resides in the Y chromosome. The sex-determining region on the Y chromosome (*SRY*) is crucial [6]: it triggers differentiation of the testis from the genital ridge and is also responsible for Leydig and Sertoli cell proliferation. In humans, *SRY* is expressed and translated in adult as well as fetal Sertoli cells and adult germ cells, although its function in adult testis is not known [7]. *SRY* expression has also been found in other tissues, such as the prostate and in prostate cancer [8]. In mice, *Sry* is active only for a very brief period between 10.5 and 12.5 days post coitum (dpc) when specific morphologic changes are observed in XY but not in the XX gonad [6, 9]. *Sry* transcripts were also found in murine adult testis, but they exist there as circular RNA molecules, in contrast to the linear form described in the genital ridge [10, 11]. The function of the protein in adult testis is still unknown; in fact the circular transcripts may not be translated at all, since no association with polysomes was found [10, 11].

Several pieces of evidence demonstrate that SRY is necessary and sufficient to induce male development. In mice, deletion of *Sry* produces XY females and hermaphrodites [12]. Only the expression of *Sry* and no other gene on the Y chromosome induces a size increase in the rudimentary XY gonad relative to the XX gonad and is responsible for cell migration from the mesonephros to the differentiating gonad [13, 14]. Moreover, the introduction of the *Sry* gene in female embryos results in the development of male gonads: these transgenic mice show normal male external genitalia and copulatory behavior, and testes with Sertoli and Leydig cells but without spermatogenic cells [15]. SRY is therefore necessary to trigger male development; however, correct male germ cell maturation does not depend on the presence of SRY.

Although extensively studied, the mode of SRY action is still uncertain. Because a DNA-binding domain of the HMG box type is present, SRY is considered to be a transcription factor belonging to the high-mobility group (HMG) family. The HMG box confers on SRY the ability to bind specific target sequences in DNA and to cause a bend in the double helix [16, 17]. 46,XY sex-reversal patients are characterized by testicular dysgenesis, ambiguous genitalia and, usually, the absence of regression of Müllerian structures (Swyer's syndrome, OMIM 306100). Genetic analysis of these patients revealed the presence of mutations in the *SRY* HMG box sequence. Although only 10–15% of patients carry mutations in *SRY*, all of these mutations save two fall in the DNA-binding domain [18, 19]. As shown by electrophoretic mobility shift assays, these mutations impair the binding of SRY protein to consensus binding sites [16].

The finding that SRY protein can induce DNA bending by angles of 60–85° [17, 20, 21] supports the idea that SRY could act as an architectural transcription factor that influences chromatin structure in regulatory regions of target genes and facilitates the assembly of regulatory complexes [22]. A confirmation of this hypothesis was the discovery of mutations in the HMG box of SRY that alter its ability to bend DNA in human XY sex-reversed females [23]. Therefore, DNA binding and bending appear to be part of the mechanism by which SRY influences transcription of genes downstream of the sex-determining cascade, either acting as a classical transcription factor or by altering chromatin structure [16, 23].

Two-hybrid screenings identified several transcription cofactors that interact with SRY. One of these proteins is the positive coactivator PC4 [24], which acts as an adaptor between the transcriptional machinery and transcription factors [25]. PC4 action is regulated by phosphorylation. SRY is able to interact only with the unphosphorylated form of PC4. SRY may bind PC4 at its consensus binding site to further recruit the transcriptional machinery [24]. After the beginning of transcription, PC4 is phosphorylated by TFIID and released from the promoter; SRY is free to bind other molecules of PC4. However, the physiological target of the SRY-PC4 complex has not been found.

The androgen receptor (AR) also interacts with SRY. The two proteins interact via the DNA-binding domain of the AR and the HMG box domain of SRY [26]. The interaction causes a marked reduction in transcriptional activity in different androgen-responsive reporter genes. AR transcriptional activity is also repressed by DAX1, a testis-expressed orphan nuclear receptor that functions as a transcriptional corepressor. The effects of SRY and DAX1 on AR activity are synergic. These results indicate that interactions between the AR, SRY, and DAX1 may contribute to normal male development [26].

Recently, another mode of regulation of SRY function was suggested by Vincent Harley and coworkers [27].

Table 1. Summary of function, reproductive phenotype and chromosomal localization of the proteins involved in male reproductive development.

Protein	Protein full name	Function	Reproductive phenotype	Chromosome position
AR	androgen receptor	binds to androgens and then stimulates transcription of androgen-responsive genes	homozygous mutants have feminized external genitalia, cryptorchidism and block in spermatogenesis	*AR: Hs* Xq11.2-q12 (locusID: 367) *Ar: Mm* X 36.0 cM (locusID: 11835)
DAX1	DSS-AHC critical region on the X chromosome, gene 1	functions as an anti-testis gene by acting antagonistically to *Sry*	*Dax1* transgenic mice show delayed testis development	*NR0B1: Hs* Xp21.3-p21.2 (locusID: 190) *Nr0b1: Mm* X B (locusID: 11614)
FGF9	fibroblast growth factor 9	involved in various biological processes during embryogenesis and adult life, including embryo implantation, morphogenesis, angiogenesis, and possibly tumorigenesis	homozygous mutants have male-to-female sex-reversal phenotype	*FGF9: Hs* 13q11-q12 (locusID: 2254) *Fgf 9: Mm* 14 21.0 cM (locusID: 14180)
GATA4	GATA-binding protein 4	transcription factor that regulates genes involved in embryogenesis and myocardial differentiation and function; mutations have been associated with cardiac septal defects	GATA 4/SF 1 cooperation for *MIS* transcription; disruption of this synergism might lead to abnormal sex differentiation	*GATA4: Hs* 8p23.1-p22 (locusID: 2626) *Gata4: Mm* 14 28.0 cM (locusID: 14463)
HSP70-2	heat shock 70-kDa protein 2	heat shock protein and molecular chaperone	homozygous male knockout mice lack postmeiotic spermatids and mature sperm, and are infertile; defects were found in the synaptonemal complexes of spermatocytes	*HSPA2: Hs* 14q24.1 (locusID: 3306) *HspA2: Mm* 12 34.0 cM (locusID: 15512)
IGF1R	insulin-like growth factor 1 receptor	plays a critical role in transformation events; highly overexpressed in most malignant tissues	triple knockout for *Igf1R*, *Ir* and *Irr* showed that insulin-like growth factor receptors are fundamental for male determination	*EGF1R: Hs* 15q25-q26 (locusID: 3480) *Egf1r: Mm* 7 33.0 cM (locusID: 16001)
IR	insulin receptor 1	regulates cell growth and proliferation; knockout mice show severe growth retardation and mild diabetes	see IGF1R	*INSR: Hs* 19p13.3-p13.2 (locusID: 3643) *Insr: Mm* 8 1.0 cM (locusID: 16337)
IRR	insulin receptor-related receptor	epidermal growth factor receptor activity; transmembrane receptor protein tyrosine kinase signaling pathway	see IGF1R	*INSRR: Hs* 1q21-q23 (locusID: 3645) *Insrr: Mm* 3 46.8 cM (locusID: 23920)
MIS	Müllerian-inhibiting substance; anti-Müllerian hormone	causes the regression of Müllerian ducts which would otherwise differentiate into the uterus and fallopian tubes	*AMH* gene is mutated in patients with persistent Müllerian duct syndrome	*AMH; Hs* 19p13.3 (locusID: 268) *Amh: Mm* 10 43.0 cM (locusID: 11705)

Table 1 (continued)

Protein	Protein full name	Function	Reproductive phenotype	Chromosome position
PC4	activated RNA polymerase II transcription cofactor 4	PC4 is a cofactor that mediates transcriptional activation of class II genes	PC4 interacts with SRY	*PC4: Hs* 5p13.3 (locusID: 10923) *Pc4: Mm* 15 A1 cM (locusID: 20024)
SF1	nuclear receptor subfamily 5, group A, member 1; steroidogenic factor 1	SF1 is involved in gonadal differentiation and steroidogenesis; knockout mice lack adrenal glands and gonads and are severely deficient in corticosterone; male and female mice have female internal genitalia, despite complete gonadal agenesis	GATA 4 and SF 1 cooperate for MIS transcription; disruption of this synergism might lead to abnormal sex determination	*NR5A1: Hs* 9q33 (locusID: 2516) *Nr5a1: Mm* 2 23.5 cM (locusID: 26423)
SIP1	SRY-interacting protein 1	Sip1 expression was detected in somatic cells and was associated with the spermatogenic activity of the testis; it interacts with the C-terminal portion of SRY	some XY sex-reversal patients lack the SRY C terminus and, perhaps, SIP1 interaction	*Sryip1: Mm* unknown (locusID: 192659)
SOX8	SRY-related HMG box 8	SOX8 and SOX9 activate transcription of the *Mis* gene through synergistic action with SF1	*Sox8* knockouts have no obvious developmental phenotype, suggesting a redundancy between SOX8 and SOX9	*SOX8: Hs* 16p13.3 (locusID: 30812) *Sox8: Mm* 17 8.0 cM (locusID: 20681)
SOX9	SRY-related HMG box 9	*SOX9* is a critical gene involved in mammalian sex determination and differentiation	*SOX9* mutants develop campomelic dysplasia; 75% of patients show sex-reversal phenotype	*SOX9: Hs* 17q24.3-q25.1 (locusID: 6662) *Sox9: Mm* 11 E2 (locusID: 20682)
SRY	sex-determining region on Y chromosome	SRY is known as the testis-determining factor (TDF), which initiates male sex determination	mutations in this gene give rise to XY females with gonadal dysgenesis	*SRY: Hs* Yp11.3 (locusID: 6736) *Sry: Mm* Y 3.0 cM (locusID: 21674)
WNT4	wingless-type MMTV integration site family, member 4	WNT4 is a secreted signaling protein; together WNT4 and DAX1 plays a concerted role in both the control of female development and the prevention of testes formation	in homozygous mutant mice, female genitalia are masculinized; mutant males are normal	*WNT4: Hs* 1p36.23-p35.1 (locusID: 54361) *Wnt4: Mm* X 4 D3 (locusID: 22417)
WT1	Wilms tumor 1	a transcription factor that functions in kidney and gonad cell proliferation and differentiation	mutations in this gene can be associated with the development of Wilms tumors in the kidney or with abnormalities of the genitourinary tract	*WT1: Hs* 11p13 (locusID: 7490) *Wt1: Mm* 2 58.00 cM (locusID: 22431)

Hs, *Homo sapiens*; *Mm*, *Mus musculus*.

SRY has two nuclear localization signals (NLSs) in its HMG domain [28]. The C- and N-terminal NLSs of SRY use different import pathways: the former (cNLS) is recognized by the nuclear import receptor protein importin (IMP) β1, the latter (nNLS) is independent of the conventional NLS-binding importins and possibly uses a novel import pathway [29]. The SRY NLS signals are highly conserved during evolution among mammals, and SRY mutated in either NLS is not efficiently transported into the cell nucleus [28, 30]. Human XY sex-reversal patients were found with mutations in these NLSs; some of these mutants were not affected in DNA binding and bending, suggesting that disruption of the cellular localization of SRY may in itself cause sex reversal [31, 32].

Two mutations were found outside the HMG box in XY sex-reversal individuals: one is a missense mutation in the N-terminal region and the other is a premature stop codon in the C-terminal region [33, 34]. The SRY-interacting protein (SIP1) was also found to interact with SRY by two-hybrid screening [35]. This protein contains two PDZ domains, and the interaction occurs between both PDZ domains and the C-terminal portion of SRY. Lack of the SRY C-terminus causes XY sex reversal as described above [34]. Even if these findings do not allow the conclusion that XY sex reversal is due to the lack of interaction between SRY and SIP1, this is an interesting hypothesis.

In mice (genus *Mus*), but not in humans, *Sry* also includes a large CAG trinucleotide repeat region encoding a C-terminal glutamine-rich domain that acts as a transcriptional trans-activator in vitro and in vivo. Truncation mutations of the *Mus musculus molossinus Sry* gene are unable to induce testis formation in XX embryos in the transgenic mouse assay, in contrast to their wild-type counterparts [36]. This raises the possibility that murine *Sry* may act via a fundamentally different biochemical mechanism compared with other mammals. Consistent with this notion, transgenic mice expressing the human SRY do not undergo XY sex reversal [37].

Although *SRY* clearly initiates male determination, so far a direct target gene has not been identified. Furthermore, SRY is not stringently conserved during evolution. Recent evidence, discussed below, leads to the conclusion that SRY is not alone in leading to maleness (see fig. 1).

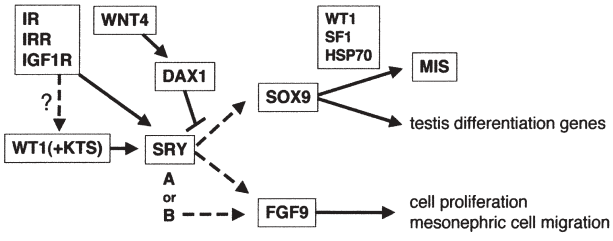

Figure 1. Schematic representation of the cascade that leads to male differentiation. (*A*) Insulin receptors function very early in the cascade that leads to male differentiation. The relationship between insulin receptors and WT1(+KTS) (dashed arrow and question mark) is still unknown. Insulin receptors and WT1(+KTS) activate expression of *SRY*; dashed arrows indicate that the relationship between *SRY-SOX9* and *SRY-FGF9* is still uncertain. SOX9 controls the expression of *MIS* and other testis differentiation genes. FGF9 governs cell proliferation and mesonephric cell migration. The blunt-ended line indicates that *DAX1* antagonizes the function of *SRY*. (*B*) An alternative interpretation of the data: *FGF9* works in parallel and not in concert with *SRY*.

SOX9

SOX9 is a member of the SOX gene family of transcription factors. SOX proteins share an amino acid sequence identity of 60% or more in their HMG box domain, which is similar to the one present in SRY (hence the name SRY-related HMG box). Human mutations in *SOX9* develop campomelic dysplasia (CD), a lethal bone malformation syndrome [2, 38]. Intriguingly, about 75% of XY CD patients show XY sex reversal [39, 40], demonstrating that *SOX9* has a central role not only in skeletal development but also in sex determination. Like SRY, the importance of DNA binding for SOX9 is revealed by the fact that more than a third of the mutations described in CD patients affect the HMG box domain. All these mutations are missense and affect the DNA-binding activity of SOX9 [41].

SOX9 is also involved in XX sex reversal. A SOX9 duplication of maternal origin was demonstrated in an SRY-negative 46,XX patient with genital ambiguity and testicular development, suggesting that an extra dose of *SOX9* is sufficient to trigger testis development in the absence of SRY [4]. Thus SOX9 obviously occupies a critical role for both the initiation and maintenance of Sertoli cell differentiation in mammals.

In mice, the expression of *Sox9* suggests a role downstream of *Sry* in Sertoli cell differentiation. Lovell-Badge and coworkers detected *Sox9* expression in the genital ridge of both sexes at 10.5 dpc, whereas Koopmann and coworkers did not find expression at the same developmental stages [42, 43]. This incongruity is most likely due to the sensitivity of the techniques used. Despite this discrepancy, both groups found that *Sox9* is up-regulated in the male genital ridge at 11.5 dpc, concomitant with a down-regulation in females. Expression is localized to the sex cords of the developing testis. Purified fetal germ cells lack SOX9, indicating that the expression of the gene is specific for Sertoli cells and does not involve germ cells. Expression is not transient, but persists throughout adulthood [42, 43].

Several studies have addressed the mode of action of SOX9. SOX9 controls the gene for the Müllerian-inhibiting substance (MIS), also known as anti-Müllerian Hormone (AMH) [44]. MIS is an essential component of male sexual differentiation [45], and is responsible for the

regression of the Müllerian ducts that are the precursors of the female reproductive organs. In mice, *Mis* is expressed in Sertoli cells concomitant with *Sox9* at 11.5 dpc; its expression increases until 19.5 dpc and dramatically decreases after birth [46, 47]. Analysis of the *Mis* promoter region revealed the presence of highly conserved binding sites for SOX9 and the orphan nuclear hormone receptor steroidogenic factor 1 (SF1), which regulates the expression of steroid hydroxylases. Transfection studies revealed that SOX9 cooperates with SF1 in the activation of *Mis* transcription. In vitro and in vivo protein-binding assays demonstrated that SOX9 and SF1 interact via the SOX9 HMG box domain and the C-terminal region of SF1 [44]. Male mice homozygous for mutation of the SF1-binding site begin the transcription of *Mis* correctly, although at reduced levels, and Müllerian ducts do regress [48]. In contrast, males homozygous for the mutation of the SOX9-binding site do not initiate *Mis* transcription, and exhibit complete retention of Müllerian duct-derived organs [40]. SOX9 is therefore essential for *Mis* transcriptional initiation, while SF1 seems to work as a modulatory/regulatory element [48]. SOX9 may bind to its binding site first and start *Mis* transcription, and then SF1 may bind its recognition site via physical interaction with SOX9. Nevertheless, *Mis* transcription appears to be more complex: recent results suggest that SOX9 may be a component of multiprotein complexes. SOX9 and the heat shock protein 70 (HSP70) interact with each other [49]; in turn, a strong interaction was found between HSP70 and Wilms tumor suppressor (WT1), which is expressed at 10.5 dpc in the urogenital ridge of both male and female animals [50, 51]. Moreover, WT1 physically interacts with SF1 to synergistically activate *Mis* transcription in vitro through the SF1-binding site [52]. Interestingly, the synergism between SF1 and WT1 can be antagonized by the nuclear receptor DAX1 through physical interaction with SF1 [53, 54]. All these data support the notion that *Mis* transcription and, more generally, the expression of many genes involved in sex determination may have multiple regulators. Recently, a closely related protein, SOX8 was described to act redundantly to SOX9. SOX8 has an expression pattern overlapping that of SOX9 during testis development, and also binds the SOX-binding site of the *Mis* promoter via a direct interaction with SF1, to synergistically enhance *Mis* expression [55].

Two publications have contributed critically to elucidating the role of SOX9 in male development. The first describes a dominant XX sex reversal in mouse due to a transgenic insertion upstream of the *Sox9* gene [56], while the second reports that ectopic expression of *Sox9* in female embryos results in the formation of male genitalia [57]. A new dominant insertional mutation, *Odsex* (*Ods*), in which XX mice carry a 150-kb deletion about 1 Mb upstream of *Sox9*, causes mice to develop as sterile XX males in the absence of *Sry* [56]. Remarkably, XX *Ods*/+ gonads at 11.5 and 14.5 dpc are histologically identical to those of normal XY male mice, and express *Sox9* – at the same stages, normal females do not express *Sox9*. Sertoli cells are not only present but also functional, as shown by *Mis* expression. Since there is no evidence for coding sequences in this region, the sequences disrupted by the insertion in the *Ods* mice might control and repress *Sox9* expression. In normal XX females, repressor molecules should inhibit *Sox9* expression by binding the regulatory elements. In normal XY males, SRY might interfere with the binding or the activity of the repressor molecules, causing *Sox9* up-regulation. In *Ods*/+ mice, the transgene insertion disrupts the regulatory region; *Sox9* is also expressed in the absence of SRY and causes dominant XX sex reversal.

To elucidate whether the expression of *Sox9* initiates the sex-reversal process or is a consequence of it, Vidal et al. [57] generated XX transgenic mice carrying the *Sox9* gene under the control of regulatory regions of the *Wt1* gene. All XX transgenic mice were phenotypically male. Male reproductive ducts developed normally and Sertoli cells were functional, as confirmed by *Mis* expression. At 13.5 dpc, testes were indistinguishable from those of wild-type XY mice; in the adult transgenic mice, testes were strongly reduced in size because of the lack of germ cells. Ectopic expression of *Sox9* is therefore sufficient to induce testis development in mice, and can substitute for *Sry* function.

The subcellular localization of SOX9 appears to be important for sex determination. SOX9 is cytoplasmic in the undifferentiated gonads of both sexes; when differentiation begins, the protein becomes nuclear in the male gonads [43, 58]. Two NLSs and one nuclear export signal (NES) have been described in the HMG box domain of SOX9 and are responsible for the nucleocytoplasmic shuttling [59, 60]. Interestingly, inhibition of nuclear export in female gonad cultures induces *Mis* expression and the formation of cord-like structures. Thus, the nuclear localization of SOX9 appears to induce male or female gonadal differentiation. Along this line of thought, the XX transgenic mice that overexpress *Sox9* might undergo sex reversal because they saturate the export mechanism of SOX9, with consequent nuclear localization of the protein [60].

Both SRY and SOX9 are clearly essential for male sex determination in mice and humans; nevertheless, they are not conserved during evolution, as would be expected for proteins with such a pivotal role. Some rodents even lack *Sry* [33]; *SOX9* is conserved in mammals and birds [43], but in alligators it is expressed only *after* testis formation, suggesting that at least in this class of vertebrates it does not determine maleness [61–63]. To complicate the issue even further, a high number of cases of human sex reversal and gonadal dysgenesis are not caused by genetic

lesions in *SRY* and *SOX9* [64, 65], suggesting that other proteins are involved in the sex determination pathway.

FGF9

FGF9 is a member of the family of fibroblast growth factors, signaling molecules known to regulate multiple developmental processes [66]. The potential role of FGF9 in male determination emerged from a recent analysis of mutant mice. *Fgf9*$^{-/-}$ mice die at birth due to lung hypoplasia [67]. At birth, most XY *Fgf9*$^{-/-}$ mice present a phenotypically female reproductive apparatus and lack testicular differentiation markers, while the rare proportion of XY *Fgf9* $^{-/-}$ phenotypic males contain poorly developed and completely disorganized testes [68]. Whether these defects are due to the reduced number and/or malfunction of Sertoli cells, or to the reduced proliferation and migration of interstitial cells, or both, remains unclear [68]. Nevertheless, there are several indications that *FGF9* could be high in the hierarchy of male sex determination genes. SRY is essential for both proliferation and migration of mesonephric cells [13, 14]. Exogenous FGF9 can induce mesonephric cell migration, and *Fgf9*$^{-/-}$ XY gonads have a reduced mesenchyme [68]. In mice, testicular *Fgf9* expression initiates at 11.5 dpc, soon after *Sry* expression and concomitant with *Sox9* expression (if we consider *Sox9* expression to begin at 11.5 dpc and not before). Some *Fgf9*$^{-/-}$ XY gonads show reduced or absent expression of *Sox9*, and some exhibit *Sox9* expression that correlates with *Mis* expression. In general, the more the phenotype is toward complete maleness, the less *Sox9* expression is affected. These findings suggest that FGF9 does not control *Sox9* expression directly, but may affect its expression by promoting testicular development. Therefore, the reciprocal position of FGF9 and SOX9 in the sex determination pathway remains to be established.

Interplay of the sex-determining genes

Sex determination is a very complex chain of events where SRY, SOX9, and FGF9 are pivotal. Nevertheless, several other molecules also play a substantial role: a short overview on these other players is given here.

Based on the existence of phenotypically male XX individuals, several authors have proposed that SRY can act as a negative regulator. WNT4 and DAX1 are a signaling molecule and a transcriptional repressor, respectively. Duplications of either *WNT4* or *DAX1* were found in XY sex-reversed patients, suggesting that they can antagonize the action of *SRY* [3, 5]. Cell transfection experiments show that WNT4 activates the transcription of *DAX1* [69, 70]. As described previously, DAX1 can bind SF1 and repress *Mis* by recruiting the corepressor NCoR [71]. Thus, DAX1 could antagonize SRY function by controlling transcription downstream of SF1. As reviewed by Clarkson and Harley [24], alternative mechanisms could be used by DAX1 to antagonize SRY function. Direct competition for DNA-binding sites was proposed on the basis of the capacity of the DAX1 DNA-binding domain to recognize DNA in the minor groove, a peculiarity shared with the HMG box site of SRY. DAX1 could also compete directly with WT1, which up-regulates SRY expression [72, 73].

WT1 also controls the expression of SRY. At least 24 different alternatively spliced isoforms have been described for WT1. Of particular interest are two alternative splice donor sites at the end of exon 9, leading to insertion or omission of three amino acids: a lysine, a threonine, and a serine (KTS); the two WT1 isoforms are denominated WT1(+KTS) and WT1(–KTS) respectively [74, 75]. Mutant mice revealed that the two isoforms play different roles in vivo [73]. Mice heterozygous for WT1(+KTS) develop normally, and adults are fertile. After 2–3 months, however, about 70% of them develop glomerulosclerosis and die because of renal insufficiency. Males homozygous for WT1(+KTS) ablation show a complete XY sex reversal and SRY transcripts are consistently reduced. Male gonads contain germ cells, albeit fewer and not properly organized. Importantly, XX gonads develop normally. Homozygous mutants that do not express the WT1(–KTS) isoform exhibit dramatic defects in both male and female gonads, whereas expression of genes of the male sex determination cascade is not affected. In Frasier syndrome patients, a mutation in the donor splice site in the exon 9 site prevents the formation of the WT1(+KTS) isoform, and these patients show similarities with the phenotype of WT1(+KTS) mutant mice: XY patients have complete sex reversal and are characterized by progressive glomerulopathy [76].

SRY, SOX9 and other SOX proteins were recently proposed as factors playing a role in pre-mRNA splicing. These proteins can bind RNA and are physically associated with spliceosomes in the nucleus [77–79]. Their ability to distort nucleic acid molecules suggests that these proteins could be involved in the splicing process, favoring and/or controlling specific splicing [79]. Thus, the molecular events that lead to gonad development could be regulated at a posttranscriptional level, as suggested by the splicing regulation of the two isoforms of WT1. Moreover, WT1(+KTS) could itself be involved in this process, since it is associated with spliceosomes and interacts with the U2AF65 splicing factor [80, 81].

Three members of the insulin receptor family of genes, *Ir* (the insulin receptor), *Igf1r* (insulin-like growth factor 1 receptor), and *Irr* (insulin receptor-related receptor), also play a role in sex determination [82]. Insulin receptors are involved in cell growth and proliferation. *Ir* knockout mice die from ketoacidosis after birth [83], *Igf1r* mutant mice have a reduced size and die at birth through respira-

tory failure [84], while *Irr* mutant mice are viable and do not show an obvious phenotype [85]. Knockouts for a single gene show no defects in male development, while male mice lacking the three genes have a completely female phenotype [82]. Male gonads do not develop, neither Sertoli nor Leydig cells form, and the expression of male-specific genes such as *Sry*, *Sox9*, and *Mis* is severely impaired. In contrast, in these chromosomally male mice, female markers are up-regulated, for example. Reduced expression of *Wnt4*, *Sry*, and *Sox9* implies that the insulin-like receptors function very early in the cascade that leads to male determination, but we do not know how.

In light of these recent findings, the insulin receptor family and WT1 should be considered at the beginning of the male determination cascade, as depicted in the model in Figure 1. WT1(-KTS) acts in the primordial genital ridge; insulin receptors and WT1 activate expression of SRY, whose function is antagonized by genes such as *WNT4* and *DAX1* and maybe others. The relationship between insulin receptors and WT1 is still unknown. At the same time or shortly later, activation by SRY and SOX9 regulates expression of testis differentiation genes like *MIS*, acting in collaboration with WT1, SF1, and HSP70. In concert or in parallel with SRY, FGF9 protein might control the size increase of the developing testis.

Transcription regulation during spermatogenesis

Male gametogenesis is a cyclic process which generates extremely differentiated cells (the spermatozoa) and involves stem cell renewal, genome reorganization and packaging, and complete cell remodeling. Problems at any of these stages may cause infertility. Gametogenesis consists of two different steps: in the first, called spermatogenesis, precursor cells (spermatogonia) are committed to two consecutive meiotic divisions with consequent reduction of genetic material; in the second, called spermiogenesis, haploid cells undergo drastic remodeling to become spermatozoa. Some of the genes essential for gamete maturation are exclusively expressed in the gonad: in general, they encode proteins involved in cell remodeling or proteins implicated in fertilization and implantation. In addition to these, other genes are ubiquitously expressed but have a fundamental role in spermatogenesis: most of them are transcription factors responsible for the expression of proteins engaged in late spermiogenesis. We will focus on these factors (summarized in table 2), and will argue that they control gene expression in testis, and are controlled themselves, in non-canonical ways. These include transcription via alternative promoters, the production of testis-specific isoforms, and alternative cellular localizations at different stages of spermatogenesis.

CREM

During spermatogenesis, several transcription factors are actively involved in germ cell-specific gene expression. Among these, the cAMP-responsive element modulator (CREM) is one of the more intriguing. CREM belongs to the CREB/CREM family of transcription factors, which participate in the response to external signals via the cyclic AMP-dependent signaling pathway. CREM is involved in several physiological functions, including memory and synaptic long-term potentiation, circadian rhythms, pituitary function, and spermatogenesis [86–89]. CREM is activated by phosphorylation of serine 117 by several kinases, principally protein kinase A (PKA), that in turn are activated by external factors such as cAMP, calcium, growth factors and stress signals [90]. CREM contains a DNA-binding domain of the bZIP type, which recognizes sequences (cAMP-responsive elements, CRE) present in the promoter of target genes, and two glutamine-rich domains flanking the phosphorylation site, which mediate protein-protein interactions. The mode of action of CREM is well characterized; once activated by phosphorylation, a conformational change allows the interaction between CREM and the CREB-binding protein (CBP). CBP is a coactivator with histone acetyltransferase (HAT) activity that probably acts both by recruiting the transcriptional machinery and by favoring chromatin remodeling [91–93] (fig. 2).

CREM is expressed at low level in all tissues, and at a high level in adult testis [94]. In testis, the *Crem* gene gives rise to a large family of transcripts via alternative splicing of exons encoding the activation domain [95]. The isoforms are not only differentially expressed during spermatogenesis but also have opposite functions. The most abundant isoform (CREMτ) is an activator of transcription; it is

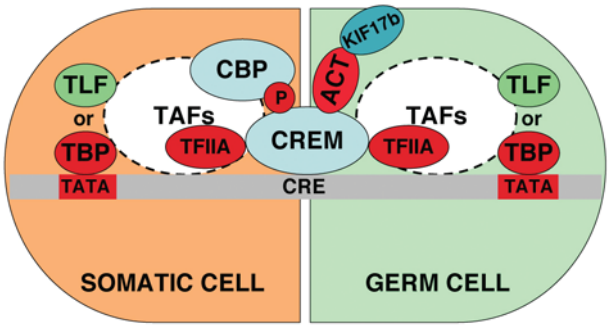

Figure 2. Mode of action of CREM in somatic and germ cells. CREM uses different mechanisms to mediate transcription of target genes: in somatic cells, CREM is activated by phosphorylation (P) and then interacts with the CREB-binding protein (CBP) that in turn recruits several factors of the general transcription machinery such as TAFs, TFIIA and TBP. In germ cells, CREM is not phosphorylated. Activation of CREM is mediated by association with the testis-specific activator of CREM (ACT). The localization of ACT in the nucleus is mediated by a kinesin (KIF17b); general transcription machinery factors are then involved in the transcription.

Table 2. Summary of function, tissue expression and chromosomal localization of the proteins involved in transcription regulation during spermatogenesis.

Protein	Protein full name	Function	Gene expression	Chromosome position
ABP	androgen-binding protein	glycoprotein secreted by Sertoli cells, that binds androgens with high affinity; transgenic mice overexpressing ABP show disrupted seminiferous tubules	Sertoli cells	*SHBG: Hs* 17p13-p12 (locusID: 6462) *Shbg: Mm* 11B3 (locusID: 20415)
ACT	activator of cAMP-responsive element modulator	activator of CREM in testis	testis specific	*FHL5: Hs* 6q16.1-q16.3 (locusID: 9457) *Fhl5: Mm* 4A3 (locusID: 57756)
ALF	TFIIA-like factor	germ cell-specific counterpart of the large (α/β) subunit of general transcription factor TFIIA that is able to stabilize the binding of TBP to DNA	testis specific	TFIIA-α/β-like factor; Hs *2p21* (locusID: 11036) *Gtf2a1lf: Mm* 17E5 (locusID: 71828)
CREM	cAMP-responsive element modulator	component of cAMP-mediated signal transduction pathway; essential during the spermatogenetic cycle; sterility in KO mice	ubiquitous expression; high expression and presence of multiple isoforms in testis	*CREM: Hs* 10p11.21 (locusID: 1390) *Crem: Mm* 18A (locusID: 12916)
ER1	estrogen receptor 1 or estrogen receptor α	nuclear hormone receptor, transcription factor	in testis is expressed in both somatic and germ cells	*ESR1: Hs* 6q25.1 (locusID: 2099) *Esr1: Mm* 10A1 (locusID: 13982)
FSC1	fibrous sheath component1	major cytoskeletal protein in the principal piece of the mammalian sperm flagellum	testis specific; expressed in round spermatids	*AKAP4: Hs* Xp11.2 (locusID: 8852) *Akap4: Mm* X 1.6 cM (locusID: 11643)
HMGB1	high-mobility group box 1	chromatin protein; works as an architectural factor facilitating the assembly of site-specific DNA-binding proteins to their cognate binding sites within chromatin; when extracellular, it promotes inflammation and cell migration	almost ubiquitous expression; in testis is expressed in spermatogonia, pachytene spermatocytes, Leydig and Sertoli cells	*HMGB1: Hs* 13q12 (locusID: 3146) *Hmgb1: Mm* 5 83.0 cM (locusID: 15289)
HMGB2	high-mobility group box 2	chromatin protein similar to HMGB1; important for germ cell maturation; subfertility in *Hmgb2* KO mice	high and widespread expression during embryogenesis, in adult tissues, high expression only in testis and lymphoid organs	*HMGB2: Hs* 4q31 (locusID: 3148) *Hmgb2: Mm* 8B2 (locusID: 97165)
HR6B	ubiquitin-conjugating enzyme E2B (RAD6 homolog)	ubiquitin-conjugating enzyme activity; testis-specific function during meiosis. *Hr6B* KO mice are infertile	ubiquitous expression	*UBE2B: Hs* 5q23-q31 (locusID: 7320) *Ube2B Mm* 11 B1.3 (locusID: 22210)
HSC70t	heat shock protein cognate 70, testis	heat shock protein with specific activity during spermatogenesis: KO males are infertile	expressed during meiosis, abundant in pachytene spermatocytes	*HSPA1L: Hs* 6p21.3 (locusID: 3305) *Hspa1l: Mm* 17 18.95 cM (locusID: 15482)
KIF17b	kinesin family member 17b	isoform of the brain-specific KIF17 kinesin; determines the intracellular localization of ACT	testis-specific expression in round and elongated spermatids	*KIF17: Hs* 1p36.12 (locusID: 57576) *Kif17: Mm* 4 D3 (locusID: 16559)

Table 2 (continued)

Protein	Protein full name	Function	Gene expression	Chromosome position
MTEST640 MTEST641 MTEST643		cDNA clones isolated from an adult mouse testis cDNA library; unknown function; abolished expression in *Tlf*$^{-}$ mice	testis specific	unknown
PRM1	protamine 1	highly basic DNA-binding protein involved in DNA packaging during spermatogenesis	mRNA in round spermatids; translation of mRNAs is delayed until the elongating stage of spermatids	*PRM1: Hs* 16p13.2 (locusID: 5619) *Prm1: Mm* 16 3.4 cM (locusID: 19118)
PRM2	protamine 2	highly basic DNA-binding protein involved in DNA packaging during spermatogenesis	mRNA in round spermatids; translation of mRNAs is delayed until the elongating stage of spermatids	*PRM2: Hs* 16p13.2 (locusID: 5620) *Prm2: Mm* 16 3.4 cM (locusID: 19119)
TBP	TATA box-binding protein	assembly and stability of the RNA polymerase II transcription preinitiation complex	ubiquitous expression; high expression and multiple isoforms in testis	*TBP: Hs* 6q27 (locusID: 6908) *Tbp: Mm* 17A2 (locusID: 21374)
TFIIAα/β and γ	transcription factor IIA	general transcription factor that stimulates RNA polymerase II-directed transcription; composed by α, β, and γ subunits	ubiquitous expression; high expression in testis	α/β subunits: *GTF2A1: Hs* 14q31.1 (locusID: 2957); *mgtf2A1: M* 12D3 (locusID: 83602) γ subunit: *GTF2A2: Hs* 15q21.3 (locusID: 2958); *Gtf2A2: Mm* chromosomal position unknown (locusID: 83601)
TIF1β	transcriptional intermediary factor 1β	transcriptional corepressor; chromatin remodeling; KO is lethal; conditional, testis-restricted KOs are infertile	ubiquitous expression; high expression in testis	*TRIM28: Hs* 19q13.4 (locusID: 10155); *Trim28: Mm* 7A2 (locusID: 21849)
TLF/TRF2	TBP-like factor; TBP-related factor 2	assembly and stability of RNA polymerase II transcription preinitiation complex; essential for gametogenesis; infertility in KO mice	ubiquitous expression; high expression in germ cells	*TBPL1*: *Hs* 6q22.1-q22.3 (locusID: 9519); *Tbpl1: Mm* 10A3 (locusID: 237336)
TP1	transition protein 1	highly basic sperm-specific nuclear protein that serves to compact DNA during late spermiogenesis	high expression in round spermatids	*TNP1: Hs* 2q35-q36 (locusID: 7141); *Tnp1: Mm* 1 38.4 cM (locusID: 21958)
TP2	transition protein 2	highly basic sperm-specific nuclear protein that serves to compact DNA during late spermiogenesis	high expression in round spermatids	*TNP2: Hs* 16p13.13 (locusID: 7142); *Tnp2: Mm* 16 3.4 cM (locusID: 21959)

Hs, *Homo sapiens*; *Mm*, *Mus musculus*; KO, knockout.

transcribed in spermatocytes and round spermatids but is translated only in round and elongated spermatids (fig. 3). The less abundant isoform (CREMβ) is expressed in premeiotic germ cells and is a repressor [95]. CREMτ promotes the expression in haploid spermatids of genes which are essential for germ cell differentiation: transition protein 1 (TP1) [96], angiotensin-converting enzyme (ACE) [97], lanosterol 14α-demethylase (CYP51) [98], and calspermin [99]. In *Crem*-deficient mice, spermatogenesis is blocked at the round spermatid stage [100, 101]; males are completely sterile and have absolutely no mature spermatozoa [100, 101]. In contrast female mice are fertile and the ovary structure is not affected, suggesting that CREM is only necessary for spermatogenesis. Moreover, CREM protein is drastically reduced or absent in human infertile patients with predominant round spermatid maturation arrest [102]. During spermiogenesis, histones are first replaced by transition proteins TP1 and TP2, whereas even smaller and more basic proteins, protamine 1 and 2, later substitute TPs and are responsible for terminal DNA compaction in mature spermatozoa. In *Crem* null mutant mice, mRNA levels of protamine 1 and 2, and TP1 and TP2, are drastically reduced [100, 101].

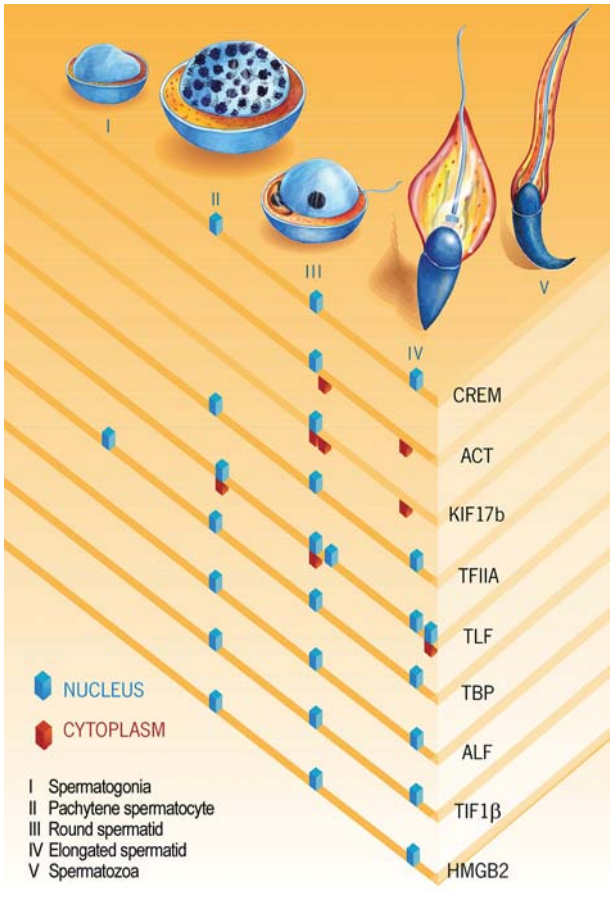

Figure 3. Protein localization during germ cell differentiation. Blue and red boxes indicate nuclear and cytoplasmic localization, respectively. When a protein is both nuclear and cytoplasmic, blue and red boxes are aligned. Conversely, boxes are slightly shifted when a protein is only nuclear or only cytoplasmic in cells of the same maturation stage. The localization of KIF17b in round spermatids is particularly complex: the protein is both nuclear and cytoplasmic in certain round spermatids, and only cytoplasmic in others.

Surprisingly, in testis, CREM is not activated via the cAMP signaling pathway. The substitution of Ser 117 to alanine does not compromise CREM-dependent transcriptional activity in haploid spermatids, suggesting that both CREM phosphorylation and CBP interaction are not necessary to activate transcription [103]. The activator of CREM in testis (ACT) was isolated by two-hybrid screening [103]. ACT is characterized by the presence of four-and-a-half LIM domains that mediate protein-protein interactions [104]. ACT has the same expression pattern as CREM, with transcript accumulation in round and elongated spermatids. In vitro and in vivo experimental approaches demonstrated that CREM and ACT associate very efficiently, and that ACT has a potent activation capacity on CREM [103]. Thus, this interaction represents a new mechanism of CREM activation which is exclusively used in male germ cells (fig. 2) [103, 105]. Interestingly, by two-hybrid screening, ACT was found to associate selectively with a novel isoform of the specific brain KIF17 kinesin, KIF17b, which is exclusively expressed in male germ cells [106]. Kinesins (KIFs) are involved in the transportation of organelles, vesicles, protein complexes and RNAs to specific destinations in the cell [107, 108]. The ACT-KIF17b interaction is restricted to specific stages of spermatogenesis and directly determines the intracellular localization of ACT. In round spermatids, KIF17b is localized in the nucleus and cytoplasm, in elongating spermatids it is both nuclear and cytoplasmic in certain cells and exclusively cytoplasmic in others. ACT is exclusively present in the nucleus of round spermatids, while in elongating spermatids, the protein is prevalently localized in the cytoplasm. By transfection assays in NIH3T3 fibroblasts and COS cells, ACT localized predominantly in the nucleus, while KIF17b had a dual localization. Interestingly, in cotransfection assays, KIF17b is able to relocalize ACT from the nucleus to the cytoplasm; inhibition of nuclear export restricts both proteins to the nucleus [106]. Therefore, kinesins can directly control the activity of a transcriptional coactivator by regulating its intracellular localization (fig. 2).

Transcription of genes by RNA polymerase II is mediated by a multiprotein complex composed of general transcription factors, such as TFIIA, -B, -D, -E, -F, and -H [109]. In tissues with intensive transcriptional activity, general transcription factors are highly expressed; in testis, TFIIA is strongly expressed [110]. The TFIIA complex consists of two ubiquitously expressed subunits, TFIIAα/β and TFIIAγ, that interact with each other and stabilize the binding of the TATA-binding protein (TBP)

to DNA. The TFIIAα/β subunit interacts with the activation domain of CREMτ [111]: the interaction does not require phosphorylation of Ser 117 and is specific to CREMτ, suggesting that the other activator isoforms use different mechanisms to interact with the target promoter. The expression profile of TFIIA in testis parallels CREMτ expression (fig. 3).

TFIIA-like factor (ALF, also known as TFIIAτ) [112, 113] is a germ cell-specific factor that can substitute the TFIIAα/β subunit and forms with TFIIAγ a functional complex that is biochemically and structurally similar to its somatic cell counterpart, and can stabilize the binding of TBP to DNA [114, 115]. During germ cell maturation, the ALF expression profile is similar to the expression pattern of TFIIA and CREM; nevertheless, CREMτ transcriptional activity does not depend on ALF since no interaction was found between CREMτ and ALF [111].

The complexity of gene regulation by CREM in germ cells makes the control of gene regulation in somatic cells look simple by comparison.

TLF

Another protein plays a specific role in transcription during spermatogenesis, TBP-like factor (TLF), also known as TRF2 (TBP-related factor). TLF is closely related to the core domain of the TATA-binding protein (TBP). DNA binding and protein-protein interactions occur via the core domain, but in contrast to TBP, TLF does not recognize the classical TATA element [116, 117]. Indeed, it may not recognize any sequence at all. Transfection experiments have shown that TLF interacts with TFIIA and acts as a repressor or activator of RNA polymerase II transcription [116, 117]. In *Caenorhabditis elegans*, *Xenopus* and zebrafish, depletion of TLF transcripts results in embryo death [118–120]. Surprisingly, TLF is not necessary for mammalian embryogenesis: TLF null mutant mice are viable but have a severe impairment of spermatogenesis [121, 122]. In *Tlf*$^{-/-}$ mice, spermatogonia and spermatocytes develop normally, and postmeiotic round spermatids are produced (fig. 3) but fail to progress to the elongated form and undergo apoptosis [121, 122]. In differentiating spermatids, the formation of the acrosome is severely impaired: enzymatic granules are positioned in the periphery and are not associated with the middle of the assembling structure; the rare elongated spermatids have vacuolated acrosomes [123].

The quantity and location of TLF fluctuate dramatically during spermatogenesis. TLF is absent in spermatogonia and primary spermatocytes, while it accumulates in pachytene spermatocytes. In round spermatids, the protein is absent at early stages and then increases and persists until the elongated spermatid stage. This peculiar expression pattern not only indicates that TLF expression is finely regulated but also that the protein must exert an important function in round spermatids. In *Tlf*$^{-/-}$ mutant mice, the expression of several genes transcribed postmeiotically is significantly reduced, for example *Tp1*, *Tp2*, *Protamine 1* and *Protamine 2*, the sperm fibrous sheath component gene *Fsc1*, and the heat shock protein *Hsc70t* [121, 122]. The expression of other testis-specific genes with unknown function, *MTEST640*, *641*, and *643* [124], was practically abolished [121]. In contrast, CREM and ACT protein expression were not impaired. These data support the hypothesis that TLF acts as a transcription factor controlling the expression of proteins necessary for the progression of gamete maturation. Alternatively, TLF could have an important role in chromatin organization. Indeed, in round spermatids, TLF is localized in the chromocenter, a structure formed by condensation of centromeric heterochromatin. Interestingly, the chromocenter is considered as an organizer for the subsequent packaging of DNA [125–127]. Early spermatids of *Tlf*$^{-/-}$ mice have a fragmented chromocenter, but not clear is whether this is a direct effect or a consequence of the lack of gene products under the control of TLF. Quite possibly, TLF might work both as a classical transcription factor and as a chromatin organizer [123].

TLF and TBP have different expression profiles in the testis. TBP is always expressed from spermatogonia to late spermatids [123]. Nevertheless, the level of TBP is regulated, and increases dramatically in pachytene spermatocytes and round spermatids; however, TBP is excluded from the chromocenter [123]. The amount of TBP expression in germ cells is so much larger than in somatic cells that it may underlie a qualitative difference in function: in adult spleen and liver there are around 0.7 and 2.3 TBP transcripts per haploid genome equivalent, respectively; in adult testis, 80–200 [110]. This is due to a modest up-regulation of the somatic promoter and to the enrolment of at least two other major and three minor promoters; ten TBP mRNA 5′ end variants have been described that differ in promoter usage and/or splicing [128].

TIF1β

The transcriptional intermediary factor β (TIF1β) is a transcriptional corepressor belonging to the large family of Krüppel-associated box (KRAB) domain-containing zinc finger proteins, important for chromatin remodeling [129–131]. TIF1β represses basal and activated transcription when tethered to DNA through fusion to a heterologous DNA-binding domain. Coimmunoprecipitation experiments showed that TIF1β is associated with heterochromatin protein 1 (HP1) α, β and γ in interphase nuclei of various mammalian cell lines [132, 133]. Null mutant embryos for TIF1β die, indicating that the protein exerts a fundamental role [134]. Conditional, testis-restricted knockouts showed that TIF1β loss results in the degeneration of seminiferous tubules [135]: some appear

morphologically normal but arrested before spermiogenesis, suggesting a premature detachment of spermatids; others are completely degenerated and have lost the typical cellular organization, with a complete absence of germ cells and severely impaired Sertoli cells, whose nuclei are distinguishable on the basal lamina while the cytoplasm is vacuolized. The third category of impaired tubules is perhaps the most interesting: it contains only mature cells while spermatogonia and spermatocytes are absent [135]. This may reflect the inability to maintain an undifferentiated, stem cell state. The expression of TIF1β is also finely regulated during spermatogenesis. TIF1β expression is high in pachytene spermatocytes and continues to the stage of elongating spermatids. In these cell types, the protein is associated with heterochromatin; in particular, in round spermatids, TIF1β is associated with the chromocenter [135]. As suggested for TLF, TIF1β might act both as a chromatin organizer (perhaps through association with HP1) and as a transcription factor.

Whether the defect in spermatogenesis is cell autonomous or not remains to be determined. In general, in a cell-autonomous phenotype, germ cells arrest at a specific maturation stage and tubules have a morphology similar to that shown for TLF- and CREM-deficient mice. In the contrast, the presence of tubules containing no germ cells but only degenerated Sertoli cells suggests that the defect is partially or totally due to an inconsistent communication between Sertoli and germ cells. Spermatogenesis is strictly dependent on communication between Sertoli and germ cells. The former nurse and help to commit germ cells to differentiation; the latter can influence Sertoli cell function and gene expression [136, 137]. *Tif1*$\beta^{-/-}$ testes are a mosaic: only a modest part of the tubules show germ cell maturation arrest, whereas the others show degeneration and vacuolization [135]. Several knockout and transgenic mice show the typical tubule disruption, consistent with alteration of Sertoli cell function. For example, depletion of the estrogen receptor (ER) [138] and overexpression of the androgen-binding protein (ABP) result in collapsed tubules containing only impaired Sertoli cells [139, 140]. Estrogens regulate androgen production, whereas ABP is a glycoprotein secreted by Sertoli cells that binds androgens produced by Leydig cells, and is proposed to regulate spermatogenesis and sperm maturation by maintaining high androgen levels in the testis and epididymis [141].

HMGB2

High-mobility group box 2 (HMGB2) is a member of the HMGB protein family, which also includes HMGB1 and HMGB3 [142, 143]. HMGB1 and HMGB2 are chromatin proteins extensively studied for their role as architectural factors. They have two DNA-binding domains of the HMG box type, which bind to the minor groove of DNA causing a local distortion of the double helix; conversely, they show high affinity for predistorted DNA. They have no sequence specificity, and are recruited to specific DNA target sequences by protein-protein interactions with several transcription factors [reviewed in refs 142, 144]. In vitro, HMGB1 and 2 are interchangeable. Their expression pattern is, however, different: HMGB1 is ubiquitously expressed from embryonic stages to almost all adult tissues (with very low expression in most of the adult brain [145]), whereas HMGB2 is highly expressed during embryogenesis and is mainly restricted to lymphoid organs and testes in the adult [146].

HMGB2 is absent in Sertoli cells and in spermatogonia. Its expression begins in spermatocytes and increases abruptly in pachytene spermatocytes and round spermatids, while it disappears in elongating spermatids and mature spermatozoa [146]. Since HMGB2 is in general a highly stable protein, its disappearance suggests that the protein is degraded in elongating spermatids. HMGB2 is restricted to the nuclei of germ cells, and associates with the chromocenter [L. Ronfani, unpublished data].

The phenotype of *Hmgb1* null mutant mice highlights the relevance of HMGB1 as a regulator of transcription. Newborn mice die within the first day of life from severe hypoglycemia and show a defect in glucocorticoid-dependent gene expression [147]. In contrast, null mutant mice for *Hmgb2* are healthy and show only reduced fertility [146]. Histology of *Hmgb2*$^{-/-}$ testes shows a mosaic effect similar to some extent to that previously described in *Tif1*$\beta^{-/-}$ and ABP transgenic mice: some tubules appear normal while some show a large degree of degeneration. Even in apparently normal tubules, gaps are present among cells and the number of apoptotic cells is increased. In others, large vacuoles are present and the regular periphery-to-lumen succession of germ cells is lost. The most degenerated tubules contain no germ cells while Sertoli cells are characterized by large vacuoles and only nuclei on the lamina are distinguishable. Thus, the absence of HMGB2 influences Sertoli cells, even if HMGB2 is not expressed in this specific cell type. Large multinucleate round cells were often present in the lumen [146]. Moreover, the acrosome is not tightly apposed to the nucleus, but separated by intervening cytoplasm. Mature spermatozoa are significantly less motile than wild-type ones and present a slightly more rounded head; however, their ability to fertilize eggs is not affected [146]. In many male mice lacking HMGB2, testes were found in the inguinal ring rather than in the natural scrotal position. Testicular descent is under control of male hormones [148, 149], and HMGB2 does interact with the AR to enhance its binding to the target sequence and its transcriptional activity [150]. However, primary mouse embryonic fibroblasts (MEFs) derived from *Hmgb2*$^{+/+}$ and *Hmgb2*$^{-/-}$ mice differ only mildly in their responses to androgens [146].

HMGB2 is therefore certainly involved in gametogenesis, although its absence does not determine complete sterility. This could be in part explained by redundancy with HMGB1. In any case, the redundancy is not complete: mice homozygous for the mutation of *Hmgb2* but wild-type for *Hmgb1* are less fertile, and some infertile human patients are characterized by the absence of HMGB2 and not of HMGB1 [151].

HR6B

Ubiquitination exerts a fundamental role in all cells in several processes such as cell division, DNA repair, apoptosis, gene expression, and stress response [152, 153]. Ubiquitination involves three different activities, performed by ubiquitin-activating (E1), -conjugating (E2), and -ligating enzymes (E3); ubiquitinated proteins are subsequently degraded by the proteasome [152, 153].

The homologs of RAD6 protein A and B (HR6A and HR6B) are nearly identical ubiquitin-conjugating enzymes, with 70% similarity to the yeast RAD6 protein [154, 155]. RAD6 is involved in several functions in the cell: N-end rule protein degradation, gene silencing, retrotransposition, postreplication DNA repair, and sporulation [156]. The protein was identified as the first ubiquitin-conjugating enzyme able to ubiquitinate histones 2A and 2B in vitro [157]; this modification is essential for sporulation and gene silencing [158, 159]. Yeast RAD6 null mutants are defective in postreplication repair, in gene silencing, and in mitotic homologous recombination [160, 161]. The mammalian homologs are expressed in several tissues and high levels of mRNA are detected in brain, heart, and testis [162]. The protein levels are similar in different cell types with the exception of the testis, where little HR6A protein is detectable in contrast to high levels of HR6B [162].

Null mutant mice for the *HR6B* gene are viable; the only detectable defect is male infertility [155]. *HR6A* null mutant mice are also viable; they do not show defects in spermatogenesis, but female infertility. There is redundancy between the two proteins as *HR6A* and *HRGB* double knockouts are not viable. [J. A. Grootegoed, W. M. Baarends, H. P. Roest and J. H. Hoeijmakers, unpublished results]

In $HR6B^{-/-}$ developing testis, spermatogenesis occurs normally while in the adult tissue, the process is severely impaired [155]. Defects begin when spermatids start to undergo nuclear condensation. Seminiferous tubules lose germ cell organization and the cytoplasm of Sertoli cells is vacuolized. Dramatically damaged mature spermatozoa are produced: 70% have an aberrant head shape and almost all are immotile. Spermatids exhibit an irregular orientation and organization, and are released prematurely in the epididymis. TP2 is present in a small number of elongated spermatids, most of which are abnormal, and is not uniformly distributed in the cell nuclei, in contrast to wild-type cells [155].

During spermatogenesis, the H2A histone is highly ubiquitinated in pachytene spermatocytes and elongating spermatids, suggesting that this modification contributes to nucleosome destabilization and probably facilitates histone to protamine replacement [163]. Surprisingly, in *HR6B* null mutant mice no alteration of the H2A ubiquitination pattern was found. Defects during spermatogenesis may be due to changes in the synaptonemal complex (SC) [163], a structure that holds paired chromosomes together during prophase I of meiosis, and is required for genetic recombination. In $HR6B^{-/-}$ pachytene spermatocytes, the SC is thinner and longer compared to the wild-type counterpart: SC proteins are lost from telomeric regions and chromatin appears less compact, maybe because of premature SC protein degradation [163]. An increased number of foci containing the mismatch DNA repair protein MLH1 (the mammalian homolog of the *Escherichia coli* MutL protein which is crucial for both meiotic recombination and postreplicative DNA mismatch repair [164, 165]) are present, reflecting a significant increase in crossing-over frequency. This increase in meiotic recombination could be due to SC modifications, likely associated with chromatin relaxation and/or the impossibility to correct DNA lesions during the last round of spermatogenic replication [163].

Transcription in spermatogenesis: a different world

We have described five different proteins that are important for correct gamete maturation. The emerging pattern is that transcription control mechanisms that govern spermatogenesis are different from those in somatic cells. In testis, ubiquitously expressed genes are transcribed from alternative, more powerful promoters, as occurs for TBP [110], or are simply overexpressed, as is the case for TFIIA [110]. In certain circumstances, testis-specific factors direct specific gene transcription. This is the case for TLF and ALF [113, 115, 121]. Activation of genes is also under the control of testis-specific cofactors. ACT mediates transcriptional activation by CREM, and neither interaction with the ubiquitous CBP protein nor phosphorylation are required [103, 105]. Moreover, localization of cofactors seems to be important, and proteins that relocalize cofactors in germ cells have been identified [106].

In mice with spermatogenesis defects, one common feature seems to be alteration of chromatin structure. Proteins with a suggested structural role are positioned in a heterochromatin zone responsible for DNA packaging, the chromocenter. Lack of these proteins determines chromocenter fragmentation and severe impairment of gametogenesis. TLF, TIF1β, and HMGB2 might just have a structural role facilitating DNA compaction or might

silence genes by sequestering them into the chromocenter [123, 135, 146]. Defects during meiosis and in the structuring of the synaptonemal complex also result in less condensed chromatin; an example are the null mutant mice for the ubiquitin-conjugating DNA repair enzyme, HR6B [155, 163].

These are only some examples of the processes that take place during spermatogenesis. Gene transcription and translation are finely regulated and use several different mechanisms that probably originated to guarantee the correct development of mature germ cells. The unraveling of all these molecular mechanisms represents a crucial checkpoint, through which we will be able to understand the causes of male infertility.

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