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Sox100B, a *Drosophila* Group E Sox-domain Gene, Is Required for Somatic Testis Differentiation

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Abstract
Sex determination mechanisms are thought to evolve rapidly and show little conservation among different animal species. For example, the critical gene on the Y chromosome, *SRY*, that determines sex in most mammals, is not found in other animals. However, a related Sox domain transcription factor, *SOX9*, is also required for testis development in mammals and exhibits male-specific gonad expression in other vertebrate species. Previously, we found that the *Drosophila* orthologue of *SOX9*, *Sox100B*, is expressed male-specifically during gonad development. We now investigate the function of *Sox100B* and find, strikingly, that *Sox100B* is essential for testis development in *Drosophila*. In *Sox100B* mutants, the adult testis is severely reduced and fails to interact with other parts of the reproductive tract, which are themselves unaffected. While a testis initially forms in *Sox100B* mutants, it fails to undergo proper morphogenesis during pupal stages, likely due to defects in the pigment cells. In contrast, no substantive defects are observed in ovary development in *Sox100B* mutant females. Thus, as is observed in mammals, a *Sox9* homolog is essential for sex-specific gonad development in *Drosophila*, suggesting that the molecular mechanisms regulating sexually dimorphic gonad development may be more conserved than previously suspected.

The development of sexually dimorphic gonads is a key outcome of sex determination programs in metazoans. In vertebrates, the formation of a testis or ovary subsequently directs the development of all other sexually dimorphic characteristics through hormonal signals (Capel, 2000). While the morphological development of the gonads is very similar in mammals, birds, and reptiles, the primary genetic mechanisms that determine sex are apparently not conserved. Vertebrates employ a wide range of methods for determining sex, ranging from a dominant male-determining gene to temperature-dependent mechanisms (Sinclair et al., 2002). Outside of the vertebrates, with the exception of the key model species *Drosophila melanogaster* and *Caenorhabditis elegans*, comparatively little is known about the molecular mechanisms that determine sex or underlie the development of the gonads. Until recently, sex determination in *Drosophila* was thought to be an entirely cell-autonomous process controlled by the balance of sets of autosomes to X chromosomes and apparently unrelated to any mechanism used by vertebrates (Cline and Meyer, 1996). Contrary to this view a number of tissues in *Drosophila* have been shown to exhibit non-autonomous sex determination, including the gonad (DeFalco et al., 2008), the genital disk (Ahmad and Baker, 2002), and the germline

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(Nothiger et al., 1989). In the case of the gonad, the sexual phenotype of some male-specific somatic cell types is independent of the internal state of the somatic sex-determination hierarchy but is influenced by sex-specific signals from other somatic gonadal cells (DeFalco et al., 2008). These observations raise the possibility that aspects of sexual differentiation may be more similar between species than hitherto suspected. Indeed, many striking parallels can be drawn between flies and vertebrates in terms of the morphological development of the gonad (Capel, 2000). Therefore, despite the apparent disparate genetic methods used to determine sex, there are tantalizing hints that the molecular switches that are responsible for the development or differentiation of the testis may be conserved.

In eutherian mammals, where sexual differentiation has been extensively studied, a picture of the molecular hierarchy controlling the sexual specification of the gonad is beginning to emerge (Bowles and Koopman, 2001; Ronfani and Bianchi, 2004). In XY individuals the presence of the Y-linked transcription factor *SRY* (Sinclair et al., 1990), in conjunction with the orphan nuclear receptor Steroidogenic Factor 1 *(SF-1)*, directly results in the up-regulation of the *SOX9* transcription factor in somatic cells of the bipotential gonad primordium (Sekido and Lovell-Badge, 2008). Together with the FGF9 growth factor, SOX9 directs somatic cells to differentiate as Sertoli cells and hence the male pathway is initiated (Sekido et al., 2004; Kim et al., 2006). In the absence of *SRY*, Wnt4 signaling represses *SOX9* and *FGF9,* resulting in female development (Kim et al., 2006). The fact that *SOX9* is necessary for normal male development and is sufficient to direct testis development in XX individuals suggests that it is the key sex determining factor downstream of *SRY* (Vidal et al., 2001; Qin and Bishop, 2005; Barrionuevo et al., 2006). While the gene network controlled by SOX9 is not yet known, there is good evidence that SOX9 collaborates with SF-1 and Wilms tumor 1 (WT1) to activate the expression of anti-Müllerian hormone (AMH). Expression of AMH results in the regression of the female ductal system, and the differentiation of a male somatic gonad ensues (De Santa Barbara et al., 1998; Nachtigal et al., 1998; Arango et al., 1999).

While the *Drosophila* genome does not contain an orthologue of *SRY*, and none of the fly *Sox* genes have expression patterns or mutant phenotypes that suggest they are involved in the canonical primary sex determination pathway (Cremazy et al., 2001), there is a single orthologue of the vertebrate group E genes *(Sox8, 9,* and *10)*, *Sox100B* (Loh and Russell, 2000). Intriguingly, we previously demonstrated that *Sox100B* expression is sexually dimorphic in embryonic gonads, marking a testis-specific group of somatic cells, the male-specific somatic gonadal precursors (msSGPs) (DeFalco et al., 2003). More recently, we reported that *Sox100B* is also expressed in another male-specific somatic gonad cell type, the pigment cells. In the embryonic precursors of the pigment cells, *Sox100B* expression and the male phenotype is induced by Wnt2 signaling from male somatic gonad precursors (SGPs) independently of the internal state of the sex-determining hierarchy in the pigment cell precursors (DeFalco et al., 2008). While we have some understanding of how the expression of *Sox100B* is controlled in the embryonic gonad, our previous studies did not allow us to assess the requirements for *Sox100B* in testis development due to the lack of suitable mutations; here we report the isolation and analysis of *Sox100B* null mutations. Strikingly, we find that in the absence of *Sox100B*, testis development is severely affected while ovarian development is comparatively normal. We find that mutations in *Sox100B* lead to the loss of a sex-specific population of somatic cells in the testis, the pigment cells, which is associated with a complete failure in gonad morphogenesis during metamorphosis. Our observations suggest that, as in vertebrates, a group E Sox protein is involved in facilitating the development of somatic cell lineages in the testis.

Materials and Methods

Drosophila

Stocks were maintained on standard cornmeal-yeast-agar at 25°C. The following stocks were used (all nomenclature is according to FlyBase) (Drysdale, 2008): dco^{j3B9} (Kloss et al., 1998); *Df(3R)tll-e/TM6B* and *Df(3R)tll-g/TM6B* (Lindsley and Zimm, 1992); *y w; P[w+ dco+]* (Zilian et al., 1999); *eve stripe 3 + 7-Gal4* (gift of S. Small); *TM3, P[Gal4-Kr.C]DC2, P[UAS-GFP.S65T]DC10*, and *CyO, P[Gal4-Kr.C]DC3, P[UAS-GFP.S65T]DC7* (Casso et al., 2000); *LacZ[B-57]* and *LacZ[842]* (Gift of E. Matunis) (Gonczy, 1995).

Genetics

To isolate deletions of *Sox100B*, the dco^{j3B9} line was crossed with a source of transposase (*Dr P[ry+ Δ2-3]99B*) (Robertson et al., 1988). Resulting *w* males were tested for viability over *Df(3R)tll-e* and 84 lethal lines analysed by Southern blotting (Loh, 1999). The 4 *Sox100B* deletion lines recovered are illustrated in figure 3A. EMS-induced alleles were isolated as follows: w^{1118} males were fed EMS and crossed to dco^{j3B9}*/TM3* females, single *w; */*dco^{j3B9} males were crossed with *w; Df(3R)j3B9-rvB* females and chromosomes lethal with this *Sox100B* deletion were tested for lethality over *Df(3R)j3B9-rv12, Df(3R)tll-e* and *Df(3R)tll-e, P[dco+]* but viability over *Df(3R)tll-g*, *P{lacW}l(3)03670*^{S020514b} (a lethal insertion in the gene distal to *dco*) and *P{SUPor-P}Gycβ100B*^{KG09937} (the next most proximal mutation).

Immunohistochemistry

Embryos, larval, pupal, and adult tissues were dissected and fixed according to standard procedures (Michaud et al., 1997; DeFalco et al., 2003). Throughout this report anti-Vasa staining is used to label the germline. The following antibodies were used: rabbit anti-Sox100B at 1:1,000 (Loh and Russell, 2000); chicken anti-Vasa at 1:5,000 (gift of K. Howard); rat anti-Vasa at 1:50 (Developmental Studies Hybridoma Bank, DSHB); rat anti-Dri at 1:200 (Gregory et al., 1996); rabbit anti-muscle myosin at 1:500 (Kiehart and Feghali, 1986); mouse anti-Abd-B[1A2E9] at 1:50, mouse anti-Eya[10H6] at 1:25; mouse anti-FasIII [7G10] at 1:30, mouse anti-Cut at 1:50 (DSHB); rabbit anti-βGal at 1:10,000 (Cappel); mouse anti-βGal at 1:10,000 (Promega); mouse anti-Orb[4H8] at 1:30 (DSHB); rabbit anti-Ems (U. Walldorf) at 1:500; mouse anti-GFP B-2 (Santa Cruz) at 1:50. Oligreen (Molecular Probes) was used to label DNA, at a 1:10,000 dilution in PBTx for 12–15 min after secondary antibody incubation and washes. Fluorescent images were collected with a Zeiss 510 Meta confocal microscope; all other images were collected with a Zeiss Axiophot microscope.

Results

Sox100B Is Dynamically Expressed throughout Testis Development

Sox100B is dynamically expressed in several tissues during embryonic development, including the gut, malpighian tubules, and gonad (Loh and Russell, 2000). With respect to the developing gonad, Sox100B is first detected in a group of mesodermal cells that originate from parasegment 13 (PS13) during late stage 11 (DeFalco et al., 2003). After the gonads coalesce at stage 15, Sox100B is restricted to a specific group of somatic cells in the male gonad, the male-specific somatic gonadal precursors (msSGPs), which are eliminated in female embryos by apoptosis (fig. 1A) (DeFalco et al., 2003). There is no expression in the germline as indicated with the germ cell marker Vasa. Tracing the lineage of the msSGPs by marking the derivatives of PS13 with a GFP marker expressed under the control of the *eve* stripe 3 + 7 enhancer (a gift from S. Small) we find that these cells give rise to the terminal epithelium of the adult testis (fig. 1B, C). The seminal vesicle, which also expresses GFP in this genetic background, is derived from the genital imaginal disc not the gonad and does not express *Sox100B*. We notice that one or two Sox100B positives cells in stage 15 embryonic gonads do not express the terminal epithelium marker (fig. 1B). Towards the end of embryogenesis, at stage 17, *Sox100B* expression has declined in the msSGPs and is now evident as specific nuclear staining in a group of large cells surrounding the testis (fig. 1D) (Hempel and Oliver, 2007; DeFalco et al., 2008). We have recently shown that these superficial cells are independent of the msSGPs and are the precursors of the pigment cells of the adult testis. Co-staining adult testes for Sox100B and a pigment cell marker (LacZ B-57) confirms that *Sox100B* is expressed in these cells (DeFalco et al., 2008). Staining larval, pupal, and adult testes for Sox100B confirms that Sox100B is maintained in the pigment cells throughout testis development (fig. 1E–J). In addition to the pigment cells, *Sox100B* is also expressed in the region of the terminal epithelium in a superficial layer of cells (fig. 1K), and we also observe weak, predominantly cytoplasmic, staining in spermatogenic cysts (not shown). *Sox100B* is not expressed in the somatic cyst cells by co-staining with the cyst marker, Eya (fig. 1L) (Fabrizio et al., 2003). Thus, in the testis, *Sox100B* is expressed predominantly in the somatic pigment cells throughout testis development.

In contrast to the expression in the male, in females we see little staining in the embryonic gonad, though a few posterior SGPs show weak, often cytoplasmic staining in some genetic backgrounds (not shown). During larval and pupal stages we observe Sox100B staining in cells that will give rise to the terminal filament of the adult ovary and this expression persists in the adult (fig. 2A, B). Somatic cells of the epithelial sheath surrounding the ovary express *Sox100B* and, as with the testis, these are distinct from Eya-expressing somatic cells (fig. 2B). Although we have not explored this expression further, these nuclei resemble epithelial sheath cells (Eya) and tracheolar cells (Sox100B) described by King et al. (1968). In the adult ovarioles we observe cytoplasmic staining in the terminal filament and the interfollicular stalk cells (fig. 2C, D). Similar to the situation in the testis, we notice transient nuclear accumulation in stage 12–13 egg chambers that becomes cytoplasmic again by stage 14 and predominantly cytoplasmic expression in follicle cells (not shown).

Isolation of Sox100B Null Mutations

Since the orthologues of *Sox100B* play a key role in vertebrate testis development, we sought to recover mutations in *Sox100B* to assess any role it may play in *Drosophila* gonad development. Starting from a pupal lethal *P*-element insertion in the *discs overgrown (dco)* gene (Kloss et al., 1998), we carried out an imprecise excision screen assaying for lethality over *Df(3R)tll-e*, a deletion uncovering the 100B region and deficient for *Sox100B* (Strecker et al., 1986). *P{lacW}dco*^{j3B9} is located within the first intron of the *dco* gene but does not affect *Sox100B* expression (fig. 3A) (Loh, 1999). We identified 4 lines carrying deletions of the *Sox100B* coding region, one of

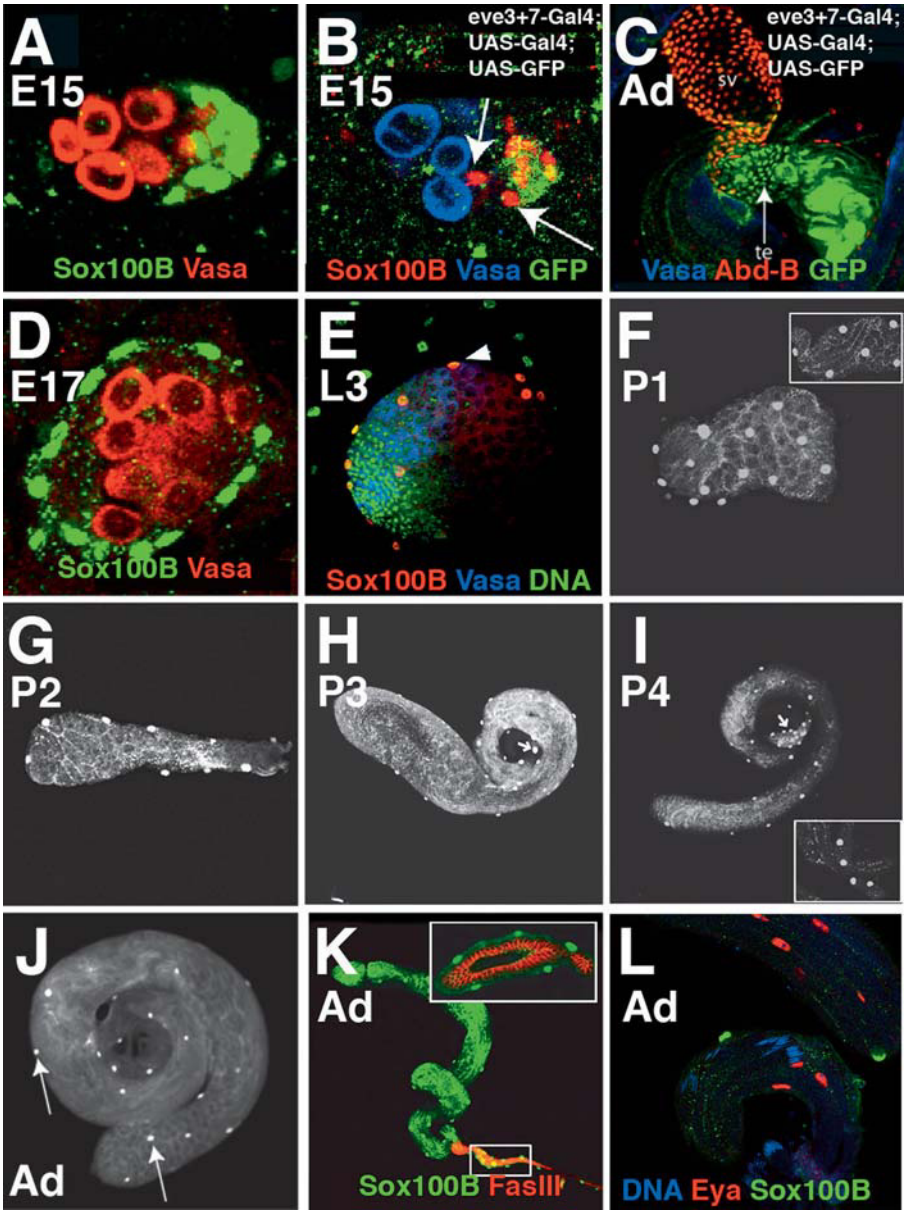

Fig. 1. Expression of *Sox100B* in the testis. In this and subsequent figures, the colors for each antibody staining are indicated by the colored text in each panel. **A** Wild type stage 15 embryonic testis stained with anti-Vasa to mark the germline and anti-Sox100B revealing the male-specific somatic gonadal precursors (msSGPs). **B** In *eve3 + 7-Gal4;UAS-Gal4;UAS-GFP* stage 15 gonads from male embryos, GFP and Sox100B mostly co-localize although some Sox100B expressing cells are negative for GFP (arrows) indicating they originate outside parasegment 13. **C** In the adult testis the GFP from the *eve3 + 7-Gal4;UAS-Gal4;UAS-GFP* reporter labels terminal epithelium (te) in addition to seminal vesicle (sv), which is also labeled with Abd-B, and other genital disc-derived structures. **D** Wild type embryonic testis at stage 17 revealing the germ line and the Sox100B positive superficial cells coating the gonad, the pigment cell precursors. **E** Third larval instar testis stained to reveal the germ line, DNA and Sox100B. Note the staining in the superficial pigment cells with their characteristic large nuclei (arrowhead). **F**–**J** Anti-Sox100B staining during testis morphogenesis: day 1 pupal testis (**F**), day 2 pupal testis (**G**), day 3 pupal testis (**H**), day 4 pupal testis (**I**), adult testis (**J**) showing the continued expression of Sox100B in pigment cells (inserts in **F** and **I**, and arrows in **J** focus on pigment cell nuclei). **K** The accumulation of Sox100B expressing cells at the basal end of the testis marks the terminal filament as revealed by staining with anti-FasIII, the insert shows an optical section demonstrating that the pigment cells lie over the terminal epithelium. **L** Sox100B does not co-localize with the somatic cyst cell marker Eya. Developmental stages of selected testes are indicated. E15, E17 = stage 15 or 17 embryos; L3 = late third instar larva; P1–4 = days of pupal development; Ad = adult.

which *(Df(3R)j3B9-rv12)* removes the entire coding sequence (fig. 3A). All 4 lines are embryonic lethal as homozygotes or in combination with *Df(3R)tll-e* and show no staining with Sox100B antisera, indicating that they are molecular null alleles of *Sox100B* (Loh, 1999). None of the deletions extend as far as the next transcription unit *(CG11317)*. Since the starting *j3B9* line is pupal lethal and the excisions are embryonic lethal, either *Sox100B* is required for aspects of embryonic development or we have generated stronger alleles of *dco*. To separate the effects of *Sox100B* from *dco* we utilized a previously described genomic *dco* rescue construct, recombining this onto the *Df(3R)tll-e* chromosome (Zilian et al., 1999). Null alleles of *dco* are viable in combination with *Df(3R)tll-e, P[dco$^+$]*, indicating effective rescue of *dco* mutations. When hemizygous with the *dco* rescue chromosome, flies carrying all 4 of the *Sox100B* deletions survive to the pharate adult stage or die shortly after eclosing from the pupal case, indicating that Sox100B is not required for embryonic viability. *Sox100B* mutant pharate adults show no discernible external morphological phenotype. More recently, we isolated a series of EMS-induced alleles that are lethal or semi-lethal in combination with *Df(3R)j3B9-rv12* and *Df(3R)tll-e, P[dco$^+$]* but viable over *Df(3R)tll-g*, which, by in situ hybridization to polytene chromosomes and Southern blotting, does not uncover *Sox100B* (supplementary fig. 1; for online supplementary figures, see www.karger.com/doi/10.1159/000200079). These alleles have similar phenotypes to the *P*-element excision lines, and 2 of the lines we tested exhibit no staining with anti-Sox100B antisera. For the subsequent analysis reported here we focused on the excision line *Df(3R)j3B9-rv12*, however, we emphasize that all of the mutations exhibit very similar phenotypes.

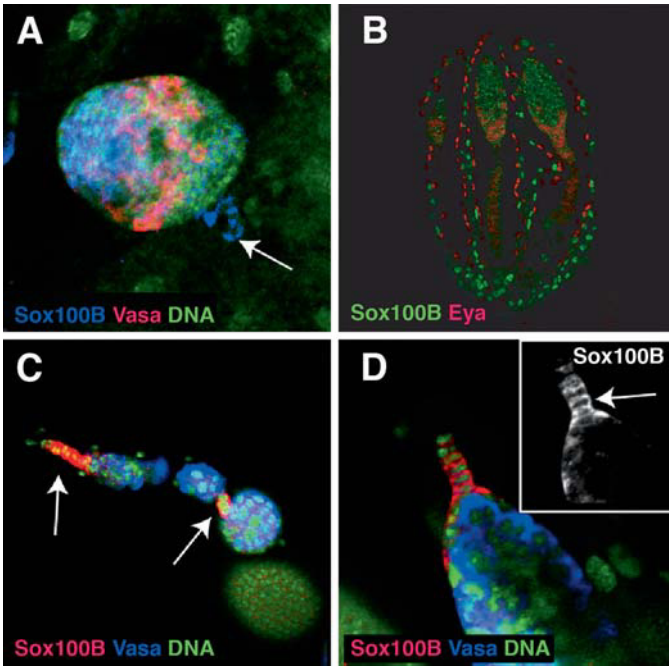

Fig. 2. Sox100B is expressed in the developing ovary. **A** Ovary from 3rd instar larva stained for Sox100B, the germline, and DNA. Sox100B expression is strongest in the anterior region, and is also present in posterior cells that may derive from adjacent fat (arrow). **B** Ovary from a Day 3 pupae (~ 76 h after puparium formation) stained for Sox100B and Eya, showing that they are expressed in distinct populations of somatic cells in the filaments and that Sox100B is cytoplasmic in the ovariole. **C**, **D** Adult wild-type ovarioles stained to reveal Sox100B, the germline, and DNA. Sox100B is observed in terminal filaments, interfollicular stalk cells (arrows) and cap cells, although staining is mostly cytoplasmic (inset in **D**).

Sox100B Mutants Fail to Develop Normal Testes but Have Normal Ovaries

We dissected *Sox100B* mutant pharate adults from the pupal case and found severe defects in the male gonad while in females the ovaries appear comparatively normal (supplementary fig. 2). In males, the portion of the reproductive tract derived from the genital imaginal disc appears normal, with fully formed seminal vesicles, accessory glands, and external wild type cuticular structures. The testes, however, are severely reduced or absent (fig. 3C, D). As with other *Sox* gene mutations (Russell et al., 1996), we noticed variability in the null allele phenotypes, with occasional individuals developing partial testis lobes (fig. 3C), however, in no cases do we observe a normal coiled testis. When partially developed testes could be isolated from pharate adults or late-stage pupae, all stages of spermatogenesis, including cysts containing apparently normal elongating sperm bundles, were observed, indicating that at least some germ cells can progress through spermatogenesis in these mutants (supplementary fig. 3). Consistent with the expression of *Sox100B* in the pigment cells through development, we find that normally pigmented regions of the reproductive tract such as the seminal vesicle are unpigmented in *Sox100B* mutants (fig. 3E, F). Staining mutant testes for the germ cell marker Vasa indicates that some germ cells are still present although they are frequently aberrantly located (fig. 3G, H). We examined markers for the terminal epithelium (Eya) and testis sheath (Abd-B and Myosin) and found that, although the sheath and terminal epithelium is somewhat disorganized, markers appear to be normally expressed. With individuals that develop a partial testis lobe, we find

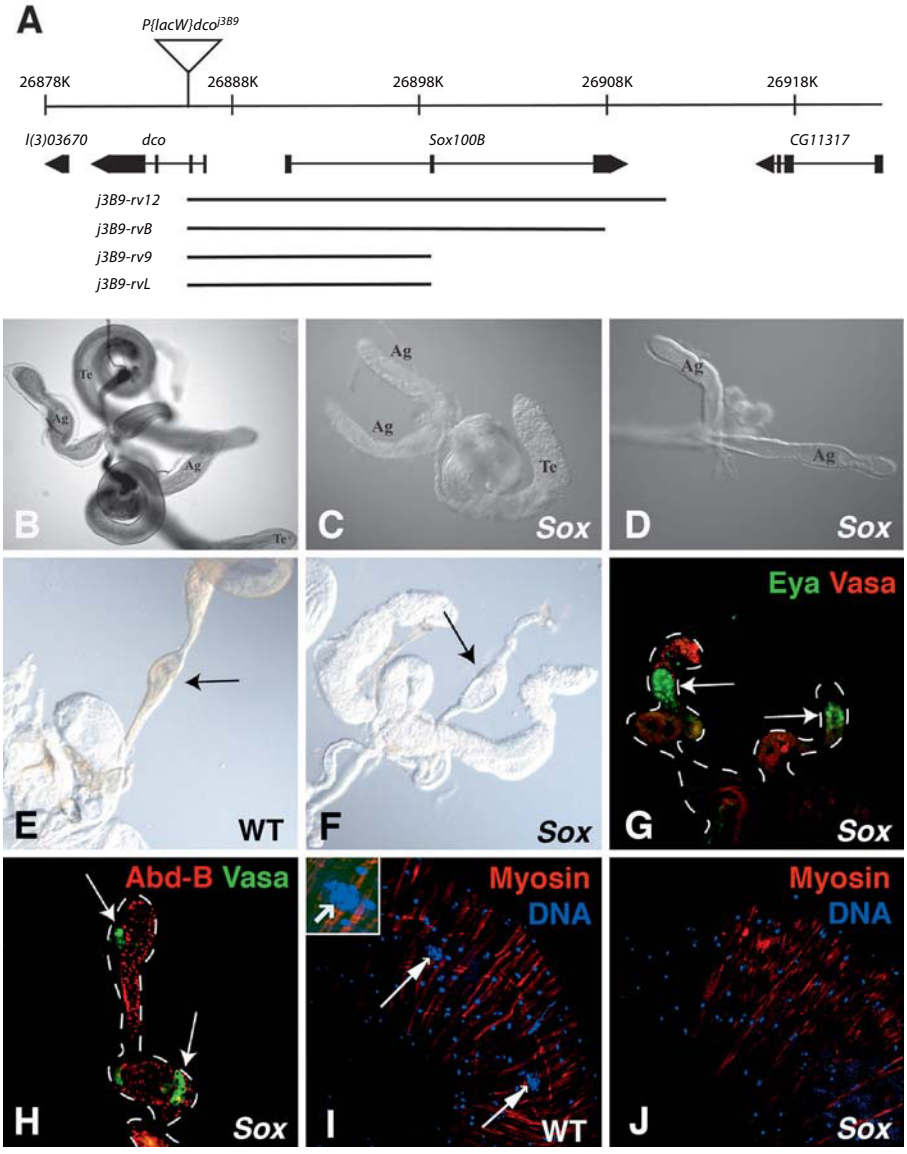

Fig. 3. Isolation of *Sox100B* mutants. **A** Diagram of part of the 100B region on chromosome arm 3R with simplified gene models derived from FlyBase; arrowheads on the last exon indicate the direction of transcription. The insertion site of the *j3B9 P*-element is indicated by the triangle. The solid lines indicate the extent of the 4 deletions. Sequence coordinates are according to release 4 of the genome sequence. **B**–**F** Dissected male reproductive tracts from adults or pharate adults. **B** In the wild type the testes (Te) are long and coiled, the accessory glands are labeled (Ag). **C** In *Sox100B* mutants, testis development is severely disrupted but occasionally a rudimentary lobe is visible. **D** Most frequently there is little testicular tissue evident, somatic derivatives of the genital disc such as the accessory glands are normal. Color micrographs of wild type (**E**) and *Sox100B* (**F**) mutant testes; the seminal vesicle (arrows) is not pigmented in the mutant. **G** Degenerated testis from a *Sox100B* mutant stained with anti-Vasa indicating the presence of very few germ cells and anti-Eya indicating the presence of rudimentary terminal epithelia. **H** Degenerated testis from *Sox100B* mutant stained with anti-Abd-B to reveal the testis sheath and with anti-Vasa to label the germline.**I**, **J** Staining for muscle myosin in wild type (**I**) and *Sox100B* (**J**) testes indicates that in rudimentary testis lobes the muscle sheath appears normal. DNA staining shows that the large nuclei characteristic of the pigment cells (arrows and insert in **I**) are absent from *Sox100B* mutant testes.

that the muscle sheath, revealed by muscle myosin staining, is comparatively normal. However, these same specimens show no signs of the large nuclei characteristic of pigment cells (fig. 3I, J). Taken together, these observations indicate that Sox100B plays a role in somatic testis morphogenesis but is not absolutely required for germline survival or correct spermatogenesis.

Although we have not carried out a detailed analysis of other tissues in *Sox100B* mutant pharate adults, internal organs such as the alimentary canal and malpighian tubules appear normal. We do note, however, that the fat body is abnormal in both male and female animals and it appears as though the larval fat has not undergone its normal histolysis (Butterworth et al., 1988; Nelliot et al., 2006). While we have not explored this area further, it is interesting to note that the pigment cells and gonadal mesoderm are derived from the same lineage as the fat body (Moore et al., 1998; DeFalco et al., 2008). We speculate that this fat defect is responsible for the pharate lethality of the *Sox100B* mutants.

Sox100B Mutants Have No Discernable Embryonic or Larval Phenotypes

Since *Sox100B* is widely expressed in the embryo and exhibits sex-specific expression in the gonad, we examined *Sox100B* mutant embryos for morphological defects

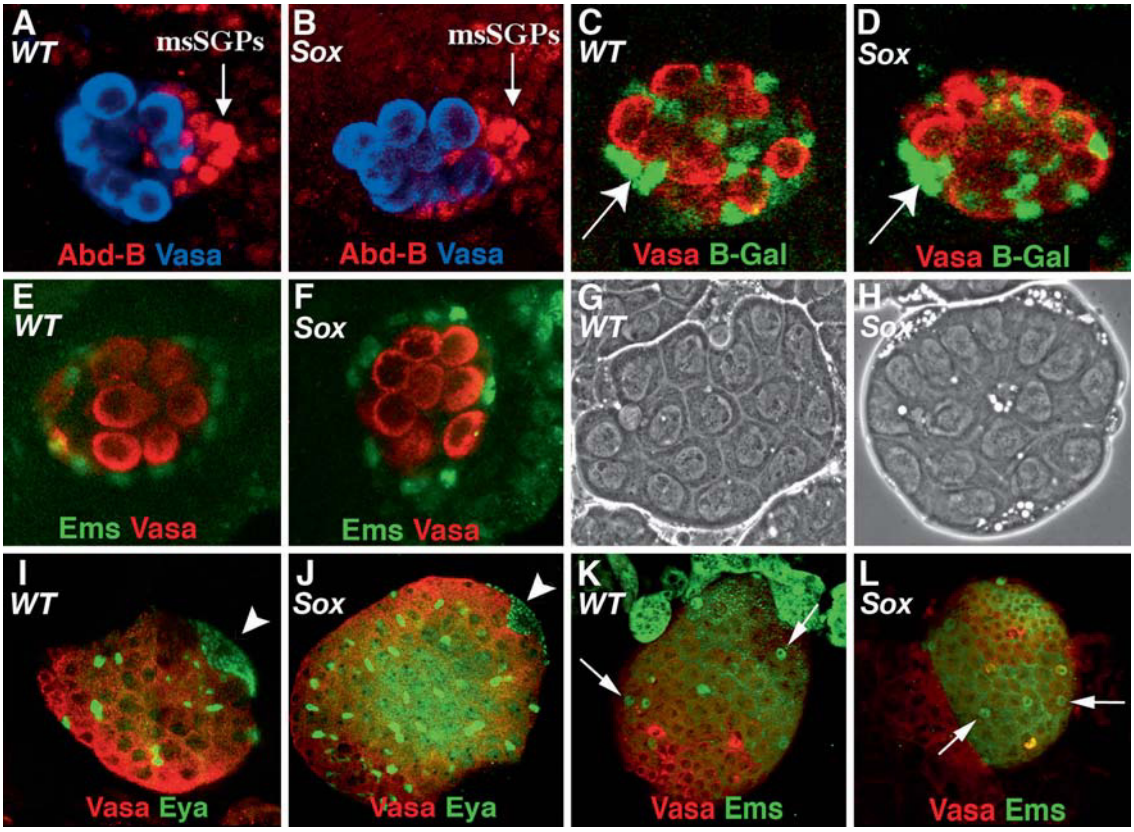

Fig. 4. Normal aspects of *Sox100B* mutant testes. **A**, **B** Stage 15 male embryonic gonads. Wild type (**A**) and *Sox100B* mutants (**B**) stained to reveal Abd-B expression in male-specific somatic gonadal precursors (msSGPs) and the germ cells. **C**–**F** Stage 17 embryonic gonads. Wild type (**C**) and *Sox100B* mutant (**D**) stained for the germline and anti-β-Gal to reveal the *LacZ[842]* enhancer trap expressed in somatic gonad precursors, including those giving rise to cyst and hub cells (arrows, hub cells). Wild type (**E**) and *Sox100B* mutant gonads (**F**) stained to reveal pigment cell precursors (Ems) showing that these cells are present in the mutants. In all cases, wild type and mutant are very similar. Note the similar Vasa staining in all cases indicating a normal germ line. The stripe of Ems expression to the right of the testis in **F** is from the CNS and unrelated to the gonad expression. **G**, **H** 16-cell spermatocyte cysts from wild type (**G**) and *Sox100B* mutant (**H**) 3rd larval instar testes. **I**–**L** Third larval instar testes from wild type (**I** and **K**) and *Sox100B* mutants (**J** and **L**) stained for the germline and for Eya indicating the presence of the msSGP-derived terminal body in *Sox100B* mutants (arrowheads). Anti-EMS staining marks the pigment cells (arrows in **K** and **L**).

or changes in marker gene expression. We have previously shown that *Sox100B* expression marks a group of sexually dimorphic somatic cells in the embryonic gonad, the msSGPs, and that deletion of *Sox100B* does not affect the initial formation of these cells (DeFalco et al., 2003). As well as *Sox100B,* the msSGPs express other markers including *Abd-B*, *Wnt2* and *eya* (DeFalco et al., 2004). In *Sox100B* null mutant embryos the expression of *Abd-B* is apparently normal (fig. 4A, B), confirming that msSGP formation is not dependent upon Sox100B function. Similarly, we find that expression of the *LacZ-842* enhancer trap, which marks the precursors of the cyst and hub cells of the testis (Gonczy et al., 1992), is also unaffected in the mutant (fig. 4C, D). We also find that Ems expression, which marks the pigment cell precursors, is unaffected in *Sox100B* mutants (fig. 4E, F) as is the expression of *Kr*-Gal4, which marks the population of fat body cells that give rise to the pigment cell precursors (data not shown). These observations indicate that Sox100B is not required for the initial formation of the gonad or for the correct expression of a range of markers associated with the precursors of the 3 major somatic components of the gonad: the msSGPs, the cyst and hub cells, or the pigment cells. Despite a complex and dynamic zygotic expression pattern for *Sox100B* in the developing embryo, and a lack of maternally contributed tran-

script or protein (Loh and Russell, 2000), we detected no defects in *Sox100B* mutants in tissues that normally express the gene (e. g., the hindgut and malpighian tubules, supplementary fig. 3) (Loh, 1999). In addition, when we examined the normal function of the malpighian tubules by imaging uric acid production in early embryos via polarized light microscopy, we found no difference between wild type and mutant (supplementary fig. 3); (Loh, 1999). Taken together, these observations indicate that *Sox100B* is not essential for the specification or morphogenesis of the embryonic tissues in which it is expressed.

We were surprised to find that the embryonic tissues expressing *Sox100B*, especially the testis, are apparently normal in the null mutant embryos and we therefore examined larval gonads. As with the embryo, we find no evidence of any defects in the mutant. Spermatogenesis is progressing normally, with wild type cysts observed in mutant testes (fig. 4G, H), which are of similar size to the wild type. Staining larval gonads for germ line (anti-Vasa) or the terminal epithelium (anti-Eya) also indicates that the mutant gonads are normal (fig. 4I, J). As in the embryo, we also find that Ems staining to mark the pigment cells is similar in wild type and mutant (fig. 4K, L). With female *Sox100B* pharate adults we find that dissected ovaries are also relatively normal (supplementary fig. 2). While we do find occasional abnormalities in the organization of the ovary, principally due to disorganization of the connective tissue and trachea covering the ovary, we emphasize that overall structure of the ovary is normal and oogenesis progresses apparently normally in most individuals. Thus, a *Drosophila* group E Sox factor has, as is the case in mammals, testis-specific mutant phenotypes.

Sox100B Mutant Testes Fail to Develop during Metamorphosis

Since the larval testis is apparently normal in *Sox100B* mutants, the defects leading to the adult phenotype must occur during metamorphosis. To investigate this, we dissected wild type and *Sox100B* mutant gonads from prepupae and pupae throughout the 4 days of metamorphosis. In the wild type, the first overt sign of differentiation is the caudal elongation of the testes as they grow to meet the somatic derivatives differentiating from the genital disc. Approximately 36 h after puparium formation, the elongating testes and genital disc derivatives meet and fuse to form the testicular duct (fig. 5A). Thereafter, the testes elongate and coil to form the mature adult organ (Bodenstein, 1950; Fuller, 1993). In contrast to the wild type, in *Sox100B* mutants we do not observe any signs of

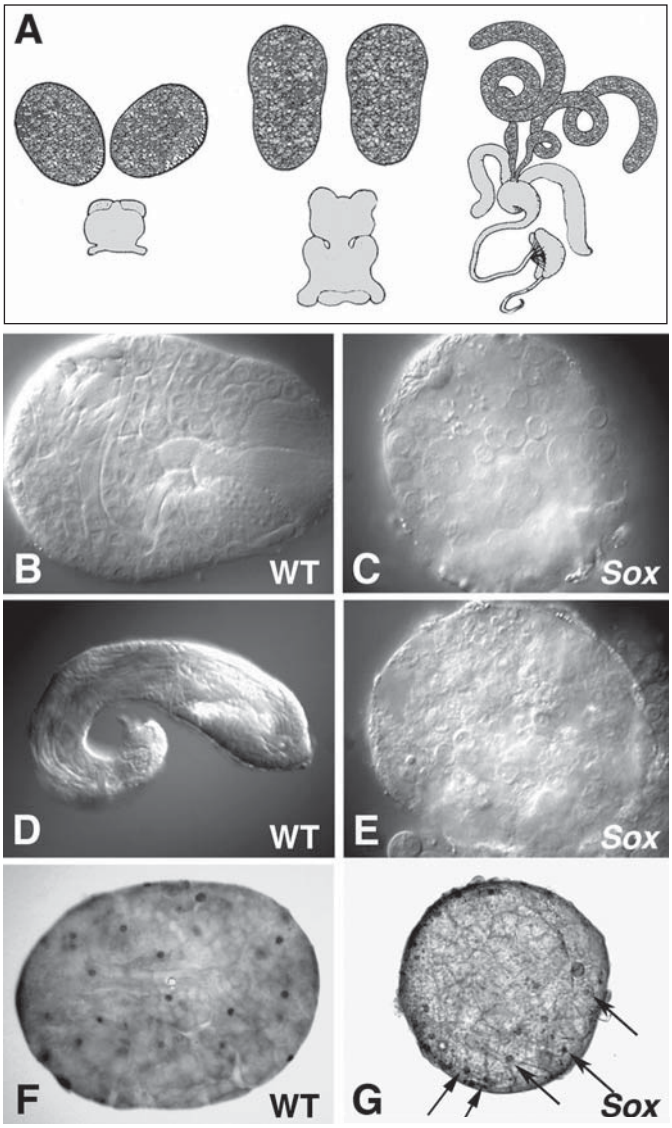

Fig. 5. *Sox100B* mutant testes fail to elongate during metamorphosis. **A** Diagram showing stages of male reproductive tract development during metamorphosis (after Bodenstein, 1950). Testis is shaded in dark grey and genital disc derivatives in light grey. At puparium formation (left) the larval testis and genital disc are distinct. At 16 h of pupal development (middle), the testes begin to elongate towards the outgrowing genital disc. The adult reproductive tract (right), showing the characteristic coiling of the testes. **B**–**G** Pupal testes: In the wild type day 1 pupae (**B**) the testis has begun to elongate whereas in *Sox100B* mutants (**C**) it remains in a larval-like state. In 2 day pupae the testis of wild type (**D**) continues to elongate while the *Sox100B* mutant still remains in a larval-like state (**E**). In wild type day 1 pupal testis, staining with anti-Ems reveals the pigment cells (**F**). In the *Sox100B* mutants, the number of Ems-positive pigment cells is considerably reduced and staining appears weaker (arrows) than in wild type (**G**).

testis elongation, rather the gonads appear to remain in the larval condition and maintain the oval shape characteristic of the larval testis throughout the pupal stages (fig. 5B–E). We stained pupal testes for the pigment cell marker EMS and found that during the 1st day of post larval life the number of EMS-positive pigment cells were reduced (fig. 5F, G). Counts of pigment cell numbers indicate that, at this stage, there is approximately half the number of EMS positive cells in the mutant compared to wild type (wild type = 24 ± 5, n = 37; mutant = 10 ± 5, n = 30). In the remaining pigment cells EMS staining is reduced compared to the wild type. Similar conclusions were reached by staining pupal testes with a DNA dye to reveal the large pigment cell nuclei (not shown). Since larval testes appear normal and express pigment cell markers but adult testes fail to develop and lack pigment cells, we conclude that, in the absence of Sox100B, pigment cells degenerate or are eliminated during the pupal stage, which is associated with a failure in testis morphogenesis.

Discussion

We show here that the *Drosophila* group E Sox-domain factor Sox100B is required for the correct differentiation of the testis. *Sox100B* mutations have relatively little effect on the development of the ovary since the ovaries are properly specified and normal oogenesis proceeds. *Sox100B* is the orthologue of the vertebrate *Sox9* gene, a key component of the sex-determining machinery in mammals that is necessary and sufficient to specify the somatic Sertoli cell lineage, which is critical for testis development (Wagner et al., 1994; Vidal et al., 2001). The intriguing finding that orthologous transcription factors control the development of male-specific somatic gonadal cell lineages necessary for development of the testis in both mammals and an arthropod suggests that aspects of the molecular hierarchy underpinning sexual differentiation may be more conserved than hitherto suspected.

In *Drosophila* the sex of the gonad is apparent from when it is formed by the coalescence of germ cells and somatic mesodermal cells (Van Doren, 2006). We have previously shown that, under the control of the somatic sex determining hierarchy, a group of male-specific gonadal mesodermal cells expressing *Sox100B* (the msSGPs) are specifically eliminated from female embryos by programmed cell death (DeFalco et al., 2003). The msSGPs give rise to the terminal epithelium of the adult testis, a structure implicated in the late stages of spermatogenesis where it plays a role in sperm coiling and release of individual sperm from the somatic cyst that encapsulates the descendants of a single germ cell as it travels through the testis (Tokuyasu et al., 1972; Fuller, 1993). Surprisingly, we found that the specification of the terminal epithelium is apparently normal in the absence of Sox100B since several molecular markers for the embryonic msSGPs and adult terminal epithelium are expressed normally in the mutants. As well as the progenitors of the terminal epithelium, we found that there are no apparent consequences of loss of *Sox100B* in most other embryonic tissues where it is normally expressed. For example, *Sox100B* is expressed from the earliest stages of hindgut development and throughout malpighian tubule development (Loh and Russell, 2000) yet these tissues appear morphologically, and in the case of the tubules functionally, normal. Consistent with the lack of phenotype, in preliminary microarray-based gene expression studies we find no significant changes in gene expression comparing *Sox100B* mutant and wild type embryos (S.N. and S.R., unpublished). The reason for the apparent dispensability of Sox100B in the embryo is currently unclear. There is no maternal contribution of transcript or protein, and redundant functions with another Sox factor are unlikely since there is little relevant overlap in *Sox* gene expression in the embryo (Cremazy et al., 2001) and *Sox100B* is the only group E *Sox* gene in the fly genome. Intriguingly, when we over-express *Sox100B* via the GAL4 system we find that it is lethal in many tissues in the embryo or larva (Loh, 1999), however, when we over-express *Sox100B* with nos-GAL4 (germ cells in embryonic and larval stages) and c587-GAL4 (in a subset of somatic cells in the ovary) (Kai and Spradling, 2003) we find mature egg chambers in the female (DeFalco, unpublished observations). This aspect of Sox100B function clearly requires further investigation by, for example, defining and analyzing Sox100B binding targets in the genome.

We recently discovered that, in addition to the msSGPs, *Sox100B* is expressed in another male-specific somatic gonad lineage, the pigment cell precursors, which are the embryonic progenitors of the pigment cells (DeFalco et al., 2008). The pigment cells form the outer layer of the testis sheath in the adult and are responsible for developing the yellow color of the testis sheath and seminal vesicle (Fuller, 1993). As we have shown, pigment cells are specified in the gonad during late embryogenesis: during the pupal stages they migrate to cover and pigment derivatives of the genital disc (Stern and Hadorn, 1939; Fung and Gowen, 1957). Wnt2 signaling is necessary for the correct development of pigment cells since

they are absent in *Wnt2* mutants although *Wnt2* is not expressed in the pigment cells (Kozopas et al., 1998). In the embryo, Wnt2 is expressed in somatic gonad precursors under the control of the sex-determining switch *doublesex* and non-autonomously specifies the pigment cell precursors from a population of cells derived from the fat body. Reception of the Wnt signal in fat body cells induces the expression of homeodomain transcription factor Empty spiracles *(ems)* that in turn activates *Sox100B* expression. In *Wnt2* or *ems* mutant embryos, pigment cell precursors are not specified and *Sox100B* is thus not expressed in pigment cell precursors but it is normally expressed in the msSGPs (DeFalco et al., 2008).

Wnt2 and *Sox100B* mutants show similar phenotypes since larval testes develop apparently normally and contain spermatocyte cysts, but adult testes are absent or rudimentary. At the onset of metamorphosis in the wild type, the testis elongates and contacts the seminal vesicles growing out from the genital disc. In *Wnt2* or *Sox100B* mutants there is an apparent block in testis development with the gonad remaining in a larval-like state, degenerating through the pupal stages to yield the severely atrophied gonads found in mutant adults. In both cases, genital disc morphogenesis appears to be normal and markers for the terminal epithelium and the muscle layer of the testis sheath are expressed, suggesting these tissues are specified normally. It is known that the muscle sheath is specified in, and derives from, the genital disc and it has been proposed that migration of muscle precursors from the genital disc to the gonad provides a signal for testis morphogenesis (Kozopas et al., 1998). In *Wnt2* mutants, pigment cells are never formed and we show that in *Sox100B* mutants the pigment cells are lost during metamorphosis. During the first day of pupal life we find less than half the normal number of pigment cells in *Sox100B* mutants, and in adults that form partial testis lobes we find no pigment cells. We therefore suggest that the failure in testis morphogenesis associated with *Wnt2* and *Sox100B* mutants is due to the absence of pigment cells. If this is the case it suggests that perhaps pigment cells are necessary for receiving or mediating the response to the muscle signal. Since in both *Wnt2* and *Sox100B* mutants the larval gonad does not elongate at all, there must either be a signal intrinsic to the gonad or it must receive a long-range signal to initiate outgrowth towards the genital disc. In this respect, the accumulation of *Sox100B* expressing cells at the basal end of the wild type testis is suggestive.

It has been known for some time now that the gonadal mesoderm and fat body share a common developmental origin (Moore et al., 1998). The finding that the pigment cell precursors are recruited from the fat body reinforces this relationship and may provide a possible avenue for exploring the lethality of *Sox100B* mutants that is common to both sexes. As we note, pharate adult *Sox100B* mutants are for the most part morphologically normal but their abdomens are full of what appears to be larval fat that has failed to undergo normal autophagy (Butterworth et al., 1988). Since we observe *Sox100B* expression in L3 larval fat body nuclei (DeFalco, unpublished observations) it suggests that Sox100B may have a role in fat metamorphosis. It will be critical to characterize the relationship between Sox100B and larval fat body in the future.

Proteins of the Sox family are defined by their homology with the HMG-box DNA-binding domain of the mammalian sex determining protein, SRY (Sinclair et al., 1990). The Sox family is a metazoan-specific class of transcription factors and in mammals there are at least 20 members that are divided into 8 subgroups based on sequence identity within and outwith the HMG-box (Soullier et al., 1999; Bowles et al., 2000; Schepers et al., 2002). In *Drosophila*, the situation is considerably simpler, with 8 *Sox* genes representing 5 subgroups (B to F) (Cremazy et al., 2001). In all animals studied to date, Sox proteins participate in a wide variety of developmental processes (Wegner, 1999) and some of these, particularly the function of group B *Sox* genes in CNS development, appear to have been conserved from flies to man (Blanco et al., 2005; Sanchez-Soriano and Russell, 1998). Sequence evidence suggests that *Sox100B* is indeed the orthologue of the vertebrate group E genes: not only is the HMG domain well-conserved but an intron splitting the HMG domain of the fly and vertebrate genes is located in exactly the same place (Bowles et al., 2000). Thus it is tempting to speculate that in a common ancestor, a group E Sox protein may have been involved in an ancient metazoan-specific process; our results clearly suggest this may have been an aspect of male gonad development.

Along with the Sox family, another group of conserved genes have been implicated in aspects of sexual dimorphism in the metazoans. Members of the *DMRT* (doublesex and Mab-3 related transcript) family are key factors involved the sex determination hierarchies of flies and worms (Raymond et al., 1998). In many vertebrates, *DMRT* homologues are expressed in the gonads (Zarkower, 2001; Hong et al., 2007) and in the mouse it is clear that *Dmrt1* plays a critical role in male gonad differentiation (Kim et al., 2007). It is therefore intriguing that, as is the case with group E Sox factors, conserved DNA-binding proteins are playing sex-specific roles in organisms as diverged as flies and mammals. Since aspects of *Sox100B*

expression in the testis are regulated by *dsx* in flies (DeFalco et al., 2003) and at least one *dsx*-related fly gene, *Dmrt93B*, is reported to be expressed in the testis according to the FlyAtlas expression database (Chintapalli et al., 2007), it will be interesting to explore whether group E Sox and Dmrt factors represent elements of a conserved testis differentiation pathway.

Our analysis shows that in *Drosophila* Sox100B has a relatively late role in testis development, but in vertebrates Sox9 acts early in the testis development pathway. While *Sox9* is required for sexual differentiation in eutherians by controlling the development of a testis-specific somatic lineage, in other vertebrates the situation is less clear. Although *Sox9* expression is sexually dimorphic in chicken, alligator and turtles, male-specific expression is only evident after the expression of anti-Müllerian hormone has been initiated (Oreal et al., 1998; Western et al., 1999; Shoemaker et al., 2007), suggesting that activation of *Sox9* is not a primary sex-determining gene in these species. In these non-eutherian vertebrates it is suggested that *Sox9* acts to control the correct differentiation of the somatic Sertoli cell lineage rather than its specification (e.g., Smith et al., 1999). While it is still possible that in these species there is differential activity of Sox9 protein that mediates specification in the gonad, there is clearly not a specific transcriptional up-regulation of *Sox9* associated with male specification as in eutherians. It is therefore possible that the ancestral function of a *Sox9*-like gene was in specific aspects of testis differentiation, a role that has been co-opted in mammals to specify sex. If this is the case, an exploration of the genes regulated by Sox100B in the fly may shed light on aspects of sex determination in man.

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