LETTERS

Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer

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The mammalian Y chromosome acts as a dominant male determinant as a result of the action of a single gene, *Sry*, whose role in sex determination is to initiate testis rather than ovary development from early bipotential gonads[1–3]. It does so by triggering the differentiation of Sertoli cells from supporting cell precursors, which would otherwise give follicle cells. The related autosomal gene *Sox9* is also known from loss-of-function mutations in mice and humans to be essential for Sertoli cell differentiation[4,5]; moreover, its abnormal expression in an XX gonad can lead to male development in the absence of *Sry*[6,7]. These genetic data, together with the finding that *Sox9* is upregulated in Sertoli cell precursors just after SRY expression begins[8,9], has led to the proposal that *Sox9* could be directly regulated by SRY. However, the mechanism by which SRY action might affect *Sox9* expression was not understood. Here we show that SRY binds to multiple elements within a *Sox9* gonad-specific enhancer in mice, and that it does so along with steroidogenic factor 1 (SF1, encoded by the gene *Nr5a1* (*Sf1*)), an orphan nuclear receptor. Mutation, co-transfection and sex-reversal studies all point to a feedforward, self-reinforcing pathway in which SF1 and SRY cooperatively upregulate *Sox9* and then, together with SF1, SOX9 also binds to the enhancer to help maintain its own expression after that of SRY has ceased. Our results open up the field, permitting further characterization of the molecular mechanisms regulating sex determination and how they have evolved, as well as how they fail in cases of sex reversal.

SRY contains a high-mobility group (HMG)-box DNA-binding domain characteristic of the SOX family of transcription factors. It is transiently expressed for a few hours in each Sertoli cell precursor between embryonic day (E)10.5 and E12.5. Since its discovery in 1990, a variety of mechanisms have been proposed by which SRY might act: first, as a repressor (or antagonist) of a repressor of male development[10]; second, through effects on local chromatin structure[11]; third, through a role in pre-mRNA splicing[12]; or fourth, as a specific transcriptional activator of one or more critical male-specific target genes, perhaps through partner proteins[13]. Moreover, several genes have been proposed as SRY targets, but so far with no evidence of a direct interaction.

Sf1 is essential in mice for the development of the gonads of both sexes from about E11.5 (ref. 14). However, SF1 seems particularly important for testis differentiation because heterozygotes can show XY female sex reversal in humans[15], and decreased expression of Sertoli cell-specific genes in mice[16]. SF1 is first expressed during mouse gonadal development at E9.5 by cells within the coelomic epithelium[17]. These give rise to daughter cells that enter the genital ridge, a proportion retaining SF1 and becoming supporting cell precursors[9]. We previously showed, using mice transgenic for a Myc-epitope-tagged *Sry* gene (Sry^{Myc}), that SRY is expressed exclusively in these precursor cells, which then show rapid upregulation of *Sox9* (ref. 9). Once SOX9 has reached a critical threshold, SRY is repressed by means of a SOX9-dependent negative feedback loop[5,9]. SF1 and SOX9 expression are subsequently maintained at high levels in Sertoli cells[17].

Sox9 is expressed in various tissues during embryogenesis[18] by means of a complex regulatory region thought to be spread over at least 1 megabase (Mb). XX sex reversal in *Odsex* mice was originally ascribed to a deletion associated with a transgene insertion about 1 Mb upstream of *Sox9*, but this is now considered to be a long-range effect of the transgene on an unidentified gonad-specific enhancer[19]. Transgenic mouse studies, with human *SOX9* YAC clones (−350 to +250 kilobases (kb))[20] or elements highly conserved between human and *Fugu SOX9* within −290 to +450 kb (ref. 21), revealed enhancers for several tissues, but not for the gonad.

We initiated our search by using a mouse *Sox9* bacterial artificial chromosome (BAC) clone (−70 to +50 kb) carrying a *lacZ* reporter (Fig. 1a). Mice transgenic for this construct (*120-lacZ*) recapitulated a significant subset of the endogenous *Sox9* expression pattern (Fig. 1c–g). Gonadal LacZ expression started shortly after E10.5 (data not shown) and increased up to E11.5 in both sexes, before becoming upregulated in the testis and downregulated in the ovary by E12.5 (Fig. 1h). Because LacZ co-localized with endogenous SOX9 in the testis, the transgene was expressed exclusively in the Sertoli cell lineage (Fig. 1h).

The 70-kb upstream sequence (*70-lacZ*) gave a similar expression pattern to that of *120-lacZ* (Supplementary Fig. 1), implying the lack of crucial tissue-specific elements within 3′ sequences. Deletions of the 70 kb revealed that 16 kb immediately 5′ of this (*16-lacZ*) still retained gonadal expression, but 7.0 kb (*7.0-lacZ*) did not (Fig. 1a). The 16-kb region was divided into four fragments (16a, 16b, 16c and 16d) and each was assayed in conjunction with the *hsp68* minimal promoter linked to *lacZ* or *CFP* (encoding cyan fluorescent protein) reporter genes. Fragments 16a and 16b, but not 16c or 16d, gave expression at E11.5 in both XX and XY genital ridges (Fig. 1b). We therefore tested the 3.2-kb region of overlap between 16a and 16b and found it sufficient for gonad-specific expression in an orientation-independent manner (Fig. 2a). It gave little, if any, expression in XX genital ridges; we therefore named it TES (for testis-specific enhancer of *Sox9*).

Because the normal level of *Sox9* expression in Sertoli cells depends on *Sry* and *Sf1* (ref. 9; data not shown), we investigated the epistatic relationship between TES and these two genes. The enhancer was active in XXSry^{Myc}, but completely inactive in XY$Sf1^{\Delta/\Delta}$ gonads (Fig. 2b, c), demonstrating that TES activity is downstream of both SRY and SF1. However, because SRY is present only transiently within Sertoli cell precursors, *Sox9* expression must be maintained by other factors, such as fibroblast growth factor 9/fibroblast growth factor receptor 2 (FGF2/FGFR2)[22,23]. This raises the possibility that the enhancer activity is a consequence of maintenance mechanisms rather than initial upregulation by SRY. We therefore backcrossed *tes-lacZ* onto mice carrying a conditional null allele of *Sox9*. TES was

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still active in homozygous XY null mutants, in cells located within the central portion of the genital ridge where SRY expression starts (Fig. 2d). At this stage it was absent from XX genital ridges, whether from $Sox9^{\Delta/\Delta}$ or wild-type animals (data not shown). As SF1 and other factors will be present in both sexes, but SRY will be XY-specific even in the absence of SOX9 (ref. 5), this proves that the enhancer is able to respond to SRY.

The question still remains whether SRY and SF1 physically interact with TES to regulate *Sox9* directly in the gonad. To address this, we performed chromatin immunoprecipitation (ChIP) assays. Twelve sets of primers spanning TES were designed to amplify any enriched fragments by PCR. In the SRY ChIP assays, we took advantage of the specific antibody against the Myc tag in Sry^{Myc} transgenic mice. There was a significant enrichment of seven fragments (IP fragments (IPFs) 2, 4, 8, 9, 10, 11 and 12) (Fig. 3a). We searched for putative SRY-binding sites (SRY-BSs) in each. Four possessed a single (IPF 12) or multiple (IPFs 2, 4 and 8) SRY-BSs, whereas three (IPFs 9, 10 and 11) contained none (Fig. 3d). In SF1 ChIP, seven fragments (IPFs 5, 6, 7, 8, 10, 11 and 12) were enriched (Fig. 3b). Each contained single (IPFs 8, 10, 11 and 12) or multiple (IPFs 5 and 7) SF1-binding sites (SF1-BSs), except for IPF 6 (Fig. 3d). Because SRY-BSs might be occupied by SOX9 after SRY expression has ceased, SOX9 ChIP was performed with E13.5 gonads. Six fragments (IPFs 5, 7, 8, 10, 11 and 12) were enriched (Fig. 3c), but it is notable that not all the fragments enriched in SRY ChIP were detected. In particular IPFs 2 and 4 were missing, suggesting that these sites are dispensable for autoregulation. IPFs 5, 7, 10 and 11, which were detected in SF1 ChIP, were also enriched even though they lack SRY-BSs (Fig. 3d), implying that SF1 bound to its BSs recruits SOX9 through protein–protein interactions[24]. The enrichment of sequences lacking either consensus site (for example IPFs 6 and 9) could be due to interactions with unidentified partner proteins, to cryptic *in vivo* binding sites, or to inefficient DNA fragmentation such that they were amplified by PCR even though the protein was bound to sites in adjacent DNA. To validate our assays further, we tested *Amh* (anti-Müllerian hormone) regulatory sequences known to be a direct target of SF1 and SOX9 *in vivo*[25] and detected specific enrichment in the testis (Fig. 3b, c).

To examine whether all the SF1- and SRY-BSs are required, we divided TES into three subfragments (EC1, EC2 and EC3) and found that gonadal expression was governed by EC2, the 1.9-kb central region (Fig. 4a). We then compared upstream sequences of *Sox9* between several vertebrate species and identified a 1.4-kb region within EC2, highly conserved in rat, dog and human (Supplementary Fig. 3). This core sequence was found to be sufficient for the enhancer activity (Fig. 4a) and named TESCO (for TES core).

To verify the significance of SRY-BSs and SF1-BSs for TESCO activity, we mutated the three consensus SRY-BSs (SRYmut), the six SF1-BSs (SF1mut) and all these sites together (BOTHmut) (Fig. 4b). Transgenic mice with SRYmut or SF1mut showed enhancer activities similar to that of the wild-type sequence, whereas those with BOTHmut showed none at all, except for one (that shown) with very weak expression (Fig. 4c). This indicates that SRY and SF1 bind TESCO directly and activate it synergistically. This synergism was quantified by co-transfection assays in COS7 cells, in which SF1 activated TESCO fivefold on its own, SRY by itself was ineffective, but a tenfold activation was observed with both factors together. This synergism was also seen in CHO and HEK-293 cells (Supplementary Fig. 4). The activity disappeared with the BOTHmut reporter construct, and almost with SF1mut, although fivefold activation by SF1 remained with SRYmut as expected (Fig. 4d). Because SF1 and SOX9 interact physically, we next tested their synergism and observed a 45-fold synergistic activation in COS7 cells (Fig. 4e). SRY and SOX9 by themselves failed to activate TESCO, despite their respective very weak and strong transactivation domains[18,26]. This was expected, because SOX protein function usually depends on partner factors. Significant levels of activation remained when either SRY-BSs or

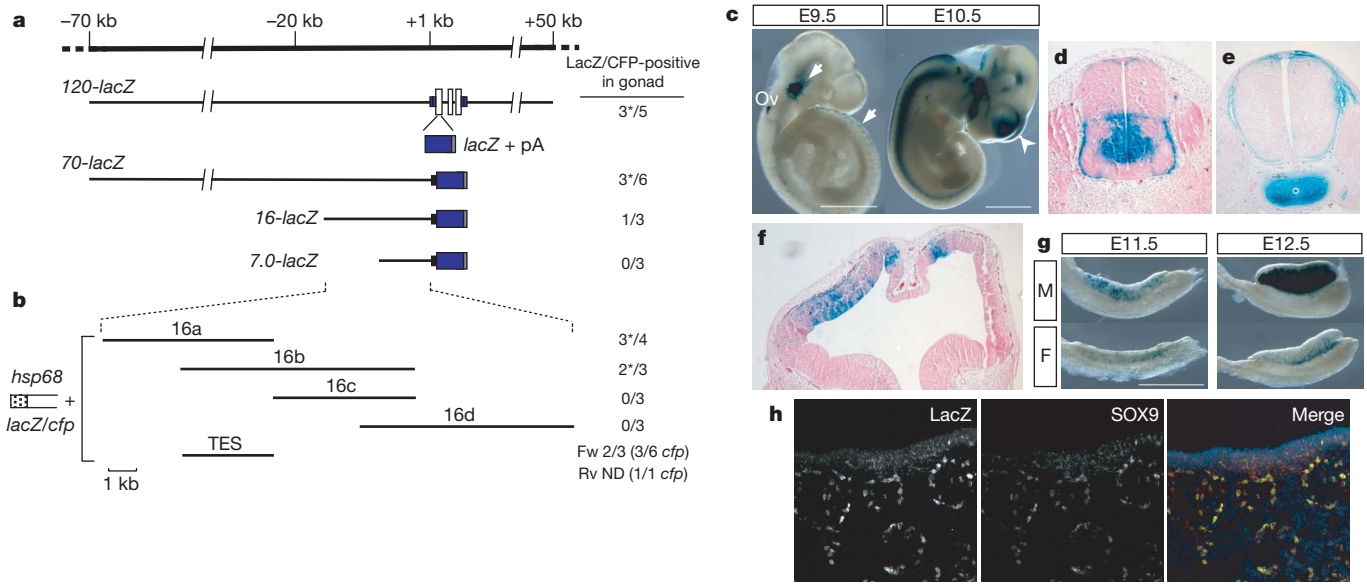

Figure 1 | Characterization of the *Sox9* locus to identify a testis-specific enhancer. a, **b**, The 120-kb BAC clone containing the *Sox9* gene, which consists of three exons (black boxes, non-coding; white boxes, coding). **a**, The reporter construct *120-lacZ* was made by replacing part of the coding region in exon 1 with *lacZ* (blue) followed by an SV40 polyadenylation signal (pA, grey). Relevant deletion constructs are shown (*70-lacZ*, *16-lacZ* and *7.0-lacZ*). **b**, Five subfragments (16a–16d and TES) assayed for their enhancer activities using *lacZ* or *cfp* reporter genes driven by the *hsp68* minimal promoter. The TES element provides testis-specific expression in both the forward (Fw) and reverse (Rv) orientations. Numbers indicate ratios of testes positive for LacZ or CFP to total transgenic lines at E12.5. ND, not determined. Asterisks: two also showed *lacZ* expression in the ovary. **c**, Expression of the *120-lacZ* transgene in whole embryos at E9.5 and E10.5. Prominent β-galactosidase activity was seen in the otic vesicle (Ov), cranial and trunk neural crest cells (arrows) and the telencephalon (arrowhead). **d**–**f**, Transverse sections through the trunk region at E10.5 (**d**) and E12.5 (**e**), counterstained with eosin, and a coronal section through the telencephalon at E11.5 (**f**) show *120-lacZ* expression in the ventral ventricular/subventricular zone of the spinal cord, sclerotomal vertebral condensation and the dorsal telencephalon, respectively. **g**, The transgene is also expressed in developing male (M) and female (F) gonads from E11.5 to E12.5. **h**, Male gonad immunostained for LacZ (left) and endogenous SOX9 (middle) shows their co-localization in Sertoli cells at E12.5 (right, yellow signals). Scale bars, 1.5 mm (**c**); 1 mm (**g**).

SF1-BSs were mutated, whereas very little activation was detected with both sets of sites mutated (Fig. 4h).

Sox9 has been found to be activated in all cases of XX male sex reversal so far examined. To examine whether the enhancer can also be activated *in vivo* in the absence of SRY, E13.5 ovaries and testes (as positive controls) from *Tesco-cfp* transgenic mice were grafted to kidney capsules of three-month-old wild-type females, where the ovaries transdifferentiate into testis-like structures[8]. As expected, CFP expression was maintained in testis grafts. Although initially negative for CFP, the transgene became active in the ovary grafts within ten days, with expression particularly evident at 30 days. Immunohistochemical analysis showed that all CFP-positive cells in the latter were positive for SF1 and SOX9, and were therefore Sertoli-like, whereas SF1 single-positive cells presumably correspond to steroidogenic cells (Supplementary Fig. 5).

These data, together with results obtained by ChIP, co-transfection assays, mutation analysis in transgenics and epistatic tests *in vivo*, support a model for the regulation of *Sox9* expression in the gonad, consisting of three phases: initiation, upregulation and maintenance (Supplementary Fig. 6). First, the initiation, which depends on SF1 but could involve other factors, need only be weak and sex-independent: it has to be sufficient to sensitize the gonad to SRY, but it must be below the threshold required to establish any additional self-reinforcing regulatory pathway. TES may not reliably reproduce the low level of *Sox9* expression seen in XX (and XY) gonads at E10.5. Nevertheless, this is seen with slightly longer fragments, which have additional SF1-BSs. WT1 is another candidate for an initiating factor because certain mutations lead to XY female sex reversal[27] and it is expressed in the supporting cell lineage, where it acts upstream of *Sf1* (ref. 28). However, WT1 and its splice variants had no effect on TESCO (data not shown). Second, SRY then acts synergistically with SF1 to upregulate *Sox9*. There may be a physical interaction between SRY and SF1, as shown previously for the HMG box of SOX9 and the carboxy-terminal domain of SF1 with respect to *Amh* regulation[24]. This could explain the enrichment of fragments lacking SRY-BS (Fig. 3d) and also previous results[29] in which mis-expression of *Sry* in transgenic mice failed to induce ectopic *Sox9* expression in SF1-negative cells. The presence of multicopy transgenes, recruitment of the other factor by protein–protein interactions and maintenance mechanisms probably account for the expression

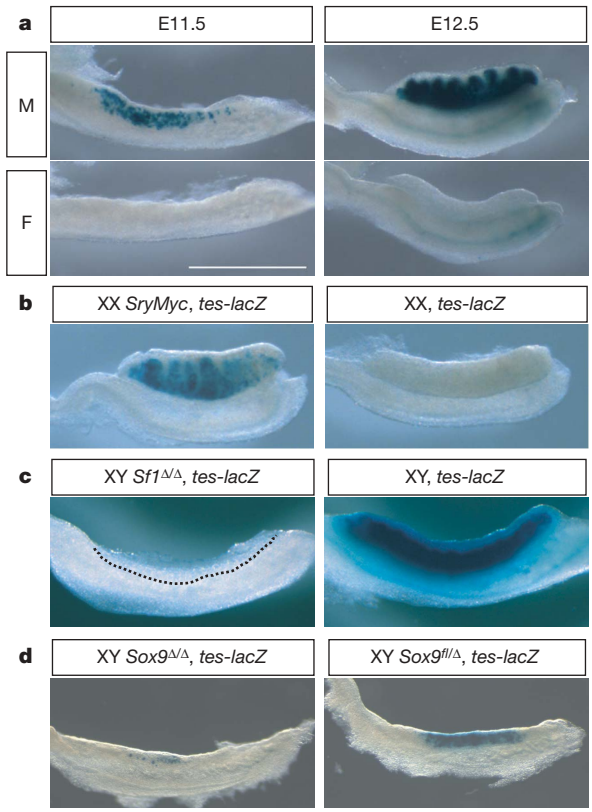

Figure 2 | Gonadal expression of *tes-lacZ* and genetic interactions with *Sry*, *Sf1* and *Sox9*. **a**, In the wild type, TES enhancer activity is detected exclusively in male gonads. **b**, **c**, The activity is induced in the gonads of XXSry^{Myc} sex-reversed mice at E12.5 (**b**, left) and is totally abolished in those of XY$Sf1^{\Delta/\Delta}$ null mutant mice at E11.5 (**c**, left). The right panels are controls. The size of the XY$Sf1^{\Delta/\Delta}$ gonad (demarcated by the dotted line) is reduced because apoptosis has started. **d**, At E11.5 in XY$Sox9^{\Delta/\Delta}$ null mutant mice, LacZ is detectable only in the central region of the gonads (left), whereas in littermates heterozygous for the flox allele (XY$Sox9^{fl/\Delta}$) it is seen throughout the gonads (right). Scale bar, 1 mm.

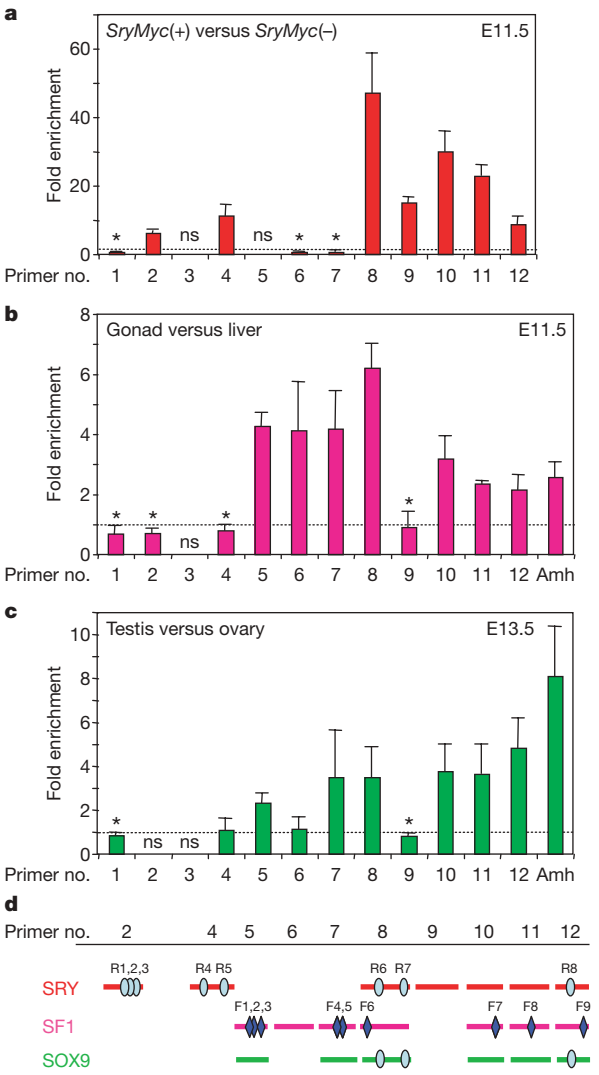

Figure 3 | Chromatin immunoprecipitation (ChIP) assays for SF1, SRY and SOX9. **a**–**c**, Quantification of fragments enriched by SRY (**a**), SF1 (**b**) and SOX9 (**c**) ChIP. An anti-Myc antibody against Myc-tagged SRY was used in SRY ChIP, whereas specific antibodies against the endogenous proteins were used in other ChIPs. Fold enrichment is expressed relative to negative control tissue, meaning that values greater than 1 represent specific enrichment. Means and s.d. from a minimum of three independent duplicate assays ($n = 6$ or more) are shown, except for Amh ($n = 4$). ns, no signal detected in either positive or negative control tissue. Asterisks, values below one (0.68–0.91) reflect site-specific backgrounds and do not imply specific enrichment in the negative control tissues. **d**, Summary of the ChIP assays. Enriched fragments are indicated by colour bars, namely red (SRY), pink (SF1) and green (SOX9). The locations of consensus SF1-BSs (F1–F9, blue diamonds) and SRY-BSs (R1–R8, light green circles) in each enriched fragment are shown. Their sequences are shown in Supplementary Fig. 2.

still seen *in vivo* when only one type of site was mutated. Third, *Sox9* requires other factors for its maintenance after SRY has ceased. An *Fgf9*/*Fgfr2* positive feedback loop has been described[22,23]. This may go through TES or TESCO (B. Capel, personal communication). There is also evidence that SOX9 may regulate SF1 expression[30], which would ensure sufficient levels of its own partner factor. In addition, we show here the likely presence of an autoregulatory loop in which SOX9 activates itself.

Although our transgenic analysis revealed an enhancer for *Sox9* that specifically recapitulates the endogenous expression pattern in the normal testis and during sex reversal, we cannot rule out the presence of additional gonad-specific enhancers, or regulatory elements interacting with TES, outside the 120-kb BAC. However, by itself TES has all the properties expected of such an enhancer. Furthermore, even though binding sites for SF1 and SRY/SOX9 are spread throughout the 3.2-kb TES, the 1.4-kb TESCO is sufficient for enhancer activity. It is possible that the additional sites are non-functional even if they are occupied by their cognate factors. Alternatively, this may reflect redundancy of binding sites as observed in other cases of transcriptional regulation. Finally, TES seems to integrate not only all the inputs into the regulation of *Sox9* required to follow a Sertoli cell fate, but also those that prevent this in ovaries, and will therefore help to define their identity and mode of action.

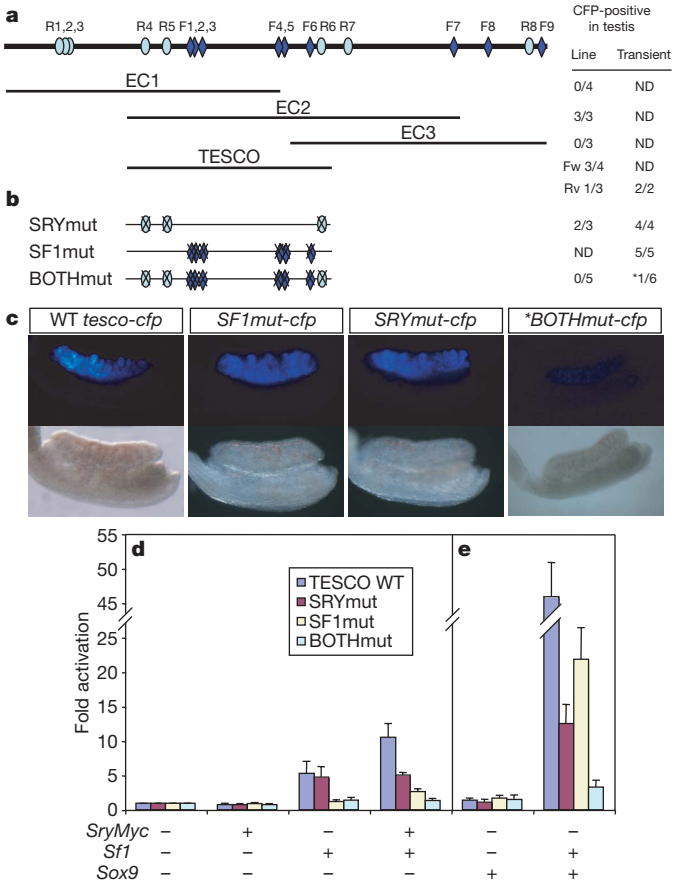

Figure 4 | Identification of the 1.4-kb enhancer core and its activation by SF1, SRY and SOX9 in a synergistic manner. a, Further deletion of TES (EC1–EC3) and comparative genome-based approaches (Supplementary Fig. 3) reveal the 1.4-kb enhancer core TESCO. **b**, Mutational analysis in transgenic mice shows no enhancer activity only when both SF1-BSs and SRY-BSs are mutated, except for one very weak sample (asterisk). Numbers indicate ratios of testes positive for CFP to total transgenics of either established lines (line) or founder embryos (transient) at E12.5. ND, not determined. **c**, CFP expression in E12.5 testes driven by wild-type and mutant TESCO sequences shown in **b**. Top, CFP fluorescence; bottom, bright field. Strong CFP fluorescence was observed with wild-type (WT) TESCO, SF1mut and SRYmut. No CFP fluorescence was detected with BOTHmut, except in one showing faint fluorescence (asterisk in **b**). **d**, **e**, *In vitro* co-transfection assays with Sry^{Myc} (**d**) or *Sox9* (**e**) in COS7 cells for quantifying mutational effects. Fold activation was calculated relative to the base level (no effectors transfected for each reporter construct). Means and s.d. from at least three independent experiments are shown.

METHODS SUMMARY

The 120-kb *Sox9* BAC clone was isolated from a 129^{SV} mouse library (Research Genetics). ChIP assays were performed with the DNA–protein complex fragmented to an average size of about 300 base pairs. In the grafting experiments, gonads dissected from *Tesco-cfp* transgenic embryos at E13.5 were grafted to the kidney capsules of three-month-old wild-type females.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions R.S. was responsible for most of the experiments, and R.L.B. for a few experiments as well as for directing the laboratory. Both planned the project and wrote the manuscript.

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ERRATUM

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Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer

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Nature 453, 930–934 (2008)

The Supplementary Methods for this Letter should have been uploaded at the time of publication. This oversight has now been rectified.

RETRACTION

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The RNA-binding protein FCA is an abscisic acid receptor

Fawzi A. Razem, Ashraf El-Kereamy, Suzanne R. Abrams & Robert D. Hill

Nature 439, 290–294 (2006)

Portions of the work repeated with respect to abscisic acid (ABA) binding have revealed errors in the calculations associated with Fig. 1, with the result that the molar ratio of ABA bound to FCA is substantially lower than claimed. There are also difficulties with the data in Fig. 2a, b that arose from the preparation of FY. We conclude that there is no effect of ABA on the FCA–FY interaction, and therefore requested to retract this paper on 14 July 2008. See the Brief Communication Arising in this issue[1].

1. Risk, J. M., Macknight, R. C. & Day, C. L. FCA does not bind abscisic acid. *Nature* doi:10.1038/nature07646 (this issue).