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# *Sox9* gene regulation and the loss of the XY/XX sex-determining mechanism in the mole vole *Ellobius lutescens*

**Stefan Bagheri-Fam · Rajini Sreenivasan · Pascal Bernard · Kevin C. Knower · Ryohei Sekido · Robin Lovell-Badge · Walter Just · Vincent R. Harley**

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**Abstract** In most mammals, the Y chromosomal *Sry* gene initiates testis formation within the bipotential gonad, resulting in male development. SRY is a transcription factor and together with SF1 it directly upregulates the expression of the pivotal sex-determining gene *Sox9* via a 1.3-kb *cis*-regulatory element (*TESCO*) which contains an evolutionarily conserved region (*ECR*) of 180 bp. Remarkably, several rodent species appear to determine sex in the absence of *Sry* and a Y chromosome, including the mole voles *Ellobius lutescens* and *Ellobius tancrei*, whereas *Ellobius fuscocapillus* of the same genus retained *Sry*. The sex-determining mechanisms in the *Sry*-negative species remain elusive. We have cloned and sequenced 1.1 kb of *E. lutescens TESCO* which shares 75% sequence identity with mouse *TESCO* indicating that testicular *Sox9* expression in *E. lutescens* might still be regulated via *TESCO*. We have also cloned and sequenced the *ECRs* of *E. tancrei* and *E. fuscocapillus*. While the three *Ellobius ECRs* are highly similar (94–97% sequence identity), they all display a 14-bp deletion (Δ14) removing a highly conserved SOX/TCF site. Introducing Δ14 into mouse *TESCO* increased both basal activity and SF1-mediated activation of *TESCO* in HEK293T cells. We propose a

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model whereby Δ14 may have triggered up-regulation of *Sox9* in XX gonads leading to destabilization of the XY/XX sex-determining mechanism in *Ellobius*. *E. lutescens/E. tancrei* and *E. fuscocapillus* could have independently stabilized their sex determination mechanisms by *Sry*-independent and *Sry*-dependent approaches, respectively.

**Keywords** Testis · *Sry* · *Sox9* · enhancer · *Ellobius* · speciation

**Abbreviations**

| | |
|---|---|
| CBX2 | Chromobox protein homolog 2 |
| DAX1 | Dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome, gene 1 |
| DMEM | Dulbecco's modified Eagle's medium |
| DMRT1 | Doublesex and mab-3-related transcription factor 1 |
| ECR | Evolutionarily conserved region |
| ETS | E-twenty-six |
| FOXL2 | Forkhead box L2 |
| HEK293 | Human embryonic kidney carcinoma |
| HMG | High mobility group |
| LEF | Lymphoid enhancer factor-1 |
| PCR | Polymerase chain reaction |
| PISRT1 | Polled intersex syndrome regulated transcript 1 |
| pRL-TK | Plasmid Renilla luciferase-thymidine kinase |
| pUC | Plasmid University of California |
| SF1 | Steroidogenic factor 1 |
| SOX | SOX = SRY-related HMG-Box |
| SRY | Sex-determining region of the Y chromosome |
| TCF | T-cell factor |
| TES | Testis-specific enhancer of *Sox9* |
| TESCO | Testis-specific enhancer of *Sox9* core |

## Introduction

Sex determination in mammals is chromosomally controlled with males and females carrying the XY and XX sex chromosomes, respectively. In most mammals, the Y chromosomal *Sry* gene triggers the fate of the bipotential gonad to develop into a testis rather than into an ovary resulting in male development (Sinclair et al. 1990; Koopman et al. 1991). The *Sry* gene evolved before the divergence of marsupials and placental mammals around 144–168 million years ago, and thus is absent in monotremes such as in platypus (Wallis et al. 2007, 2008). The SRY protein is the founder member of the SOX (SOX = SRY-related high mobility group (HMG)-Box) family of transcription factors which share at least 50% homology within their DNA-binding and bending HMG domain (Bowles et al. 2000; Schepers et al. 2002). Despite its pivotal role in mammalian male sex determination, a handful of rodent species have been identified who apparently determine sex in the absence of *Sry* and a Y chromosome. These include the mole voles *Ellobius lutescens* and *Ellobius tancrei* (Matthey 1958; Just et al. 1995; Vogel et al. 1998) and the spiny rats *Tokudaia osimensis* and *Tokudaia tokunoshimensis* (Soullier et al. 1998). The loss of the Y chromosome in these two genera can be considered as independent events because *Ellobius* and *Tokudaia* belong to different subfamilies, to Arvecolinae and Murinae, respectively. Their sex-determining mechanisms, i.e. which gene acts as the sex-determining switch, remain elusive. However, it has been demonstrated recently that males of both *Sry*-negative *Tokudaia* species have additional copies of *Cbx2*, a gene acting upstream of *Sry* (Katoh-Fukui et al. 1998), suggesting that CBX2 might be involved in male sex determination in *Tokudaia* (Kuroiwa et al. 2011). In *E. lutescens*, several genes have been excluded as the sex-determining switch genes namely *Dax1*, *Sf1*, *Foxl2/Pisrt1*, *Sox9*, *Sox3* and *Dmrt1* (reviewed in Just et al. 2007).

The genus *Ellobius* contains at least five species, namely *Ellobius fuscocapillus*, *E. lutescens*, the sister species *E. tancrei* and *Ellobius talpinus*, and *Ellobius alaicus* (Just et al. 2007; Romanenko et al. 2007). The only species of this genus with an XY/XX karyotype and for which the presence of the *Sry* gene could be demonstrated is *E. fuscocapillus* (Just et al. 1995). *E. lutescens* has an odd number of chromosomes, with both sexes having the karyotype 2n=17,X (Matthey 1958). The sister species *E. tancrei* and *E. talpinus* have an even number of chromosomes ranging from 2n=32,XX to 2n=54,XX and a constant number of 2n=54, respectively, in both sexes (Kolomiets et al. 1991; Romanenko et al. 2007). Since the *Sry* gene is present in *E. fuscocapillus*, it can be assumed that the common ancestor of these *Ellobius* species also possessed *Sry*, but that *Sry* was lost shortly after or during the speciation of *Ellobius*.

Evolution of new species can be driven by various genetic mechanisms, including gene mutations and gene

duplications resulting in novel protein functions, gene loss and mutations in *cis*-regulatory elements altering gene regulation. A recent breakthrough in the field of mammalian sex determination was the finding that in the mouse, SRY up-regulates the Sertoli cell expressed gene *Sox9* through direct binding to a 1.3-kb *cis*-regulatory element, termed *TESCO* (testis-specific enhancer of *Sox9* core), which is located 13 kb upstream of *Sox9* (Sekido and Lovell-Badge 2008). Like *Sry*, *Sox9* is both required and sufficient for male sex determination, for example, XX transgenic mice over-expressing *Sox9* develop as infertile males (Foster et al. 1994; Wagner et al. 1994; Vidal et al. 2001; Chaboissier et al. 2004; Barrionuevo et al. 2006). It is therefore possible that *Sox9* is the only important target for SRY in the developing testis. The discovery of the testis-specific enhancer of *Sox9* now opens the possibility to screen *Sry*-negative mammals for sequence variations in *TESCO*. This analysis might reveal clues as to which transcription factors bind to *TESCO* in those species and might shed light on the fundamentals of SRY–*TESCO* interaction. The genus *Ellobius* is a bona fide model for such analyses since closely related species exist with *Sry* (*E. fuscocapillus*) and without *Sry* (*E. lutescens*).

## Materials and methods

### Cloning of *Ellobius TESCO* sequences

The 2.2-kb fragment of *E. lutescens* was amplified by polymerase chain reaction (PCR) using the human forward primer *hSox9TE2F:* 5′-TTAGCAGAAATCAGCTGTAATA-3′ and the mouse reverse primer *mSox9TE1R*: 5′-CCTTTAGGGGTAAAAACC-3′ (Fig. 1a). PCR reactions were performed in a total volume of 25 μl, using 50 ng DNA sample per reaction. Cycling conditions were 95°C for 3 min, and 35 cycles at 95°C (30 s), 46°C (30 s) and 72°C (3 min). The evolutionarily conserved region (*ECRs*; 200 bp genomic fragments) of *E. lutescens*, *E. tancrei*, *E. talpinus* and *E. fuscocapillus* were amplified by PCR using human forward primer *hSox9TE2F*: 5′-TTAGCAGAAATCAGCTGTAATA-3′ and the *E. lutescens* reverse primer *eSox9TE3R*: 5′-CCTCCCTGTTGTTGGTAGCTGCC-3′ (Fig. 1a). Cycling conditions were 95°C for 3 min, and 35 cycles at 95°C (30 s), 57°C (30 s) and 72°C (1 min). The 2.2-kb *E. lutescens* and the 200-bp *E. fuscocapillus* fragments were cloned into the pGEM®T Easy vector (Invitrogen) according to the manufacturer's instructions.

### DNA sequence analysis

Four pGEM®T Easy clones containing the 2.2-kb *E. lutescens* fragment and two pGEM®T Easy clones containing the 200-bp *E. fuscocapillus* fragment were sequenced with standard vector primers Sp6 and T7, using an Applied Biosystems 3130xl Genetic Analyzer fitted with an 80-cm array to generate read lengths of approximately 1,000 bases. The four pGEM®T Easy clones containing the 2.2-kb *E. lutescens* fragment were also sequenced with the internal primers *NestF*: 5′-AGCAAGGCAGGACTCAGACA-3′ and *NestR*: 5′-ATCCGGTCCAGCATTCACCT-3′. The *ECRs*

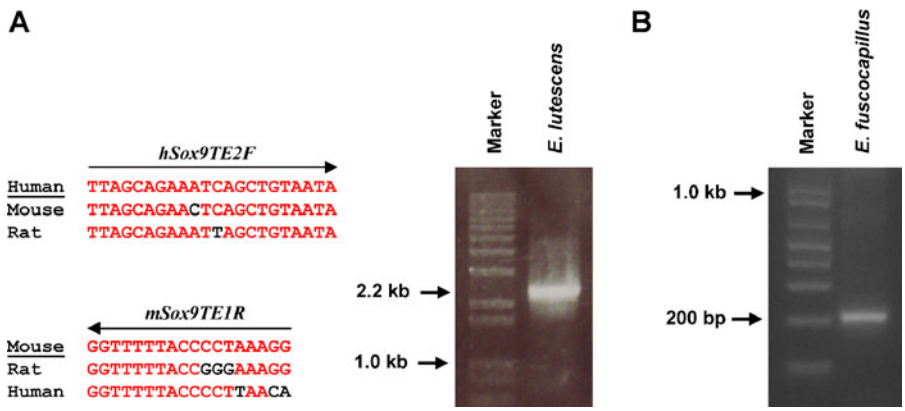


**Fig. 1** Amplification of *Ellobius TESCO* sequences by genomic PCR. **a** Amplification of a 2.2-kb genomic fragment from *Ellobius lutescens* by PCR using human primer *hSox9TE2F* and mouse primer *mSox9TE1R*. Nucleotides which are conserved in at least two species are in *red*. **b** Amplification of a 200-bp genomic fragment from *Ellobius fuscocapillus* by PCR using the human primer *hSox9TE2F* and the *E. lutescens* primer *eSox9TE3R*

(200 bp PCR fragment) from five *E. lutescens*, two *E. tancrei* and three *E. talpinus* individuals were amplified and sequenced using primers *hSox9TE2F* and *eSox9TE3R*.

### Identification of transcription factor binding sites

To identify putative transcription factor binding sites within the *ECRs* of *E. lutescens*, *E. tancrei* and *E. fuscocapillus*, we used the online program MatInspector (www.genomatix.de) with core and matrix similarity set to 1.00 and optimized, respectively. The MatInspector library at the time of analysis was the Matrix Family Library version 8.4 (June 2011).

### Site-directed mutagenesis

The *TESCO*-E1b-Luc reporter was constructed by cloning the mouse 1.3-kb testis-specific enhancer of *Sox9* (*TESCO*) (Sekido and Lovell-Badge 2008) by PCR into the E1b-luciferase reporter (Bernard et al. 2008). The *TESCO-Δ14*-E1b-Luc construct was generated by site-directed mutagenesis (Stratagene Kit) of the *TESCO*-E1b-Luc construct using primers SOXDELMF: 5′-CACAAAATAACAATGCCTTCTGGC TAAGAAAGAGAAGACTCC-3′ and SOXDELMR: 5′-GGAGTCTTCTCTTTCTTAGCCAGAAGG CATTGTTATTTTGTG-3′ according to the manufacturer's instructions.

### In vitro luciferase assays

Human embryonic kidney carcinoma (HEK293T) cells were cultured at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM), high glucose, GlutaMAX media (Invitrogen) containing 10% foetal bovine serum, 1% sodium pyruvate and 1% penicillin-streptomycin. For in vitro luciferase assays, 30,000 cells were seeded into each well of a 96-well tissue culture plate 24 h prior to transfection. Cells in each well were co-transfected with the reporter construct *TESCO*-Δ14-E1b-Luc (10 ng), *TESCO*-E1b-Luc (10 ng) or the empty vector E1b-Luc (8 ng), together with 40 ng of each of the expression constructs pCDNA3-SF1, pCDNA3-SRY or pCDNA3-SOX9. pRL-TK-Renilla (Promega; 1 ng) was included as an internal control. Total DNA amount was made up to 100 ng per well using the plasmids pCDNA3 and pUC. The cells were transfected with FuGENE6 Transfection Reagent (Roche) following the manufacturer's instructions. Cell lysis was performed 46 h after transfection, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity (Luc) was normalised against that of Renilla luciferase (Ren). Five independent assays were performed, each in triplicate. Paired *t* tests were performed for statistical analysis.

## Results

### Cloning of the *ECR*s of *E. lutescens*, *E. tancrei*, *E. talpinus* and *E. fuscocapillus*

Previously, we identified an *ECR* of 180 bp within the 1.3-kb mouse *TESCO* sequence present in mammals, birds, reptiles and amphibians (Bagheri-Fam et al. 2010). The *ECR* contains five highly conserved modules (*ECRi-v*) with putative binding sites for SOX, T-cell factor (TCF)/lymphoid enhancer factor-1 (LEF), GATA and other transcription factors. These modules were spared from sequence variation throughout evolution, implying that they might be important for *Sox9* regulation in the testis. Indeed, module *ECRii* contains the SOX site R5 (AACAAT), one of three SOX sites important for SRY/SOX9-mediated activation of *TESCO* in vitro and in vivo in the mouse (Sekido and Lovell-Badge 2008). We aimed to clone and sequence the *ECR* from *E. lutescens* (*Sry*-negative) and *E. fuscocapillus* (*Sry*-positive), which might reveal clues how *Sry* was lost in *E. lutescens*. Since genomic sequences from *Ellobius* are currently not available, we designed primer pairs within human and mouse *TES* for genomic regions highly conserved across species. Using the primer pair *hSox9TE2F*/*mSox9TE1R*, we were able to amplify a 2.2-kb genomic fragment from *E. lutescens* by PCR (Fig. 1a), which was subsequently cloned into the pGEM®T Easy vector for sequencing. The 2.2-kb genomic fragment contains the *ECR* and includes all but the first 0.2 kb of mouse *TESCO*. Comparison between the *E. lutescens* sequence and mouse *TESCO* using the online tool CLUSTAL (http://www.ch.embnet.org/software/ClustalW.html) revealed 75% sequence identity over 1,135 bp (Fig. S1). Over the same region, the evolutionarily more closely related species mouse and rat and the evolutionarily more

distantly related species mouse and human show 83% and 60% sequence identity, respectively (data not shown). This demonstrates that *TESCO* is highly conserved in *E. lutescens* despite the loss of *Sry*. We were not able to amplify the corresponding 2.2-kb genomic fragment of *E. fuscocapillus* using primer pair *hSox9TE2F*/*mSox9TE1R*, possibly due to primer mismatches. However, a 200-bp genomic fragment containing the *E. fuscocapillus ECR* was successfully amplified by PCR (Fig. 1b) using the human primer *hSox9TE2F* and the *E. lutescens* specific primer *eSox9TE3R*. Using the same primer pair, we also amplified the *ECRs* from the *Sry*-negative species *E. tancrei* and of the closely related *E. talpinus* which were identical in sequence (Fig. 2).

## Identification of a 14-bp deletion (Δ14) within the *ECR* of *Ellobius*

The *Ellobius ECR* sequences spanning from *ECRi* to *ECRv* were aligned with those of human, mouse, rat, opossum, platypus, chicken, lizard and frog (Bagheri-Fam et al. 2010) (Fig. 2). The *ECRs* of *E. lutescens/E. fuscocapillus* and of *E. tancrei/E. fuscocapillus* share a sequence identity of 94% and 97%, respectively, while the mouse/rat and mouse/human *ECR* sequences are 88% and 81% identical, respectively (Table 1). This shows that the *ECRs* between the *Sry*-negative *Ellobius* species and the *Sry*-positive *E. fuscocapillus* are highly conserved with no major sequence rearrangements (Fig. 2).

Notably, all *Ellobius* species carry a large deletion within the *ECR* (14 bp when compared to mouse/rat (Δ14) and 15 bp when compared to other vertebrates), which removes most of the evolutionarily conserved module *ECRiii* including its SOX/TCF site, as well as additional 3′-flanking sequences (Fig. 2, highlighted in cyan). In the remainder of module *ECRiii*, *E. lutescens* shows a unique T to C change. The 14-bp deletion in *Ellobius* is an intriguing finding since no deletions in module *ECRiii* are found in the *ECR* sequences of 41 vertebrate species (including 37 mammalian species) obtained from the *nucleotide collection* (nr/nt), *high throughput genomic sequences* (htgs) and *whole-genome shotgun reads* (wgs) databases at the National Centre of Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Moreover, only two of the 37 mammalian species show sequence variation in *ECRiii*, namely the Chinese hamster (CTTTCAG to CTTTGGA) and the bottlenose dolphin (CTTTCAG to CTTTTAG). Another noteworthy sequence variation present in all *Ellobius* species is a C to A change in module *ECRv*.

Only *E. fuscocapillus* shows an A to G change in module *ECRii*, predicted to disrupt the SOX site R5 (Fig. 2, highlighted in cyan) which is important for SRY and SOX9-mediated activation of *TESCO* in the mouse (Sekido and Lovell-Badge 2008). In all *Ellobius* species, no sequence variation was found in modules *ECRi* (SOX/TCF site) and *ECRiv* (GATA site; Fig. 2).

## Sequence variation in the *Ellobius ECR*s alter the prediction of potential transcription factor binding sites

To investigate whether the *Ellobius* sequence variations in modules *ECRii*, *ECRiii* and *ECRv* influence the prediction of potential transcription factor binding

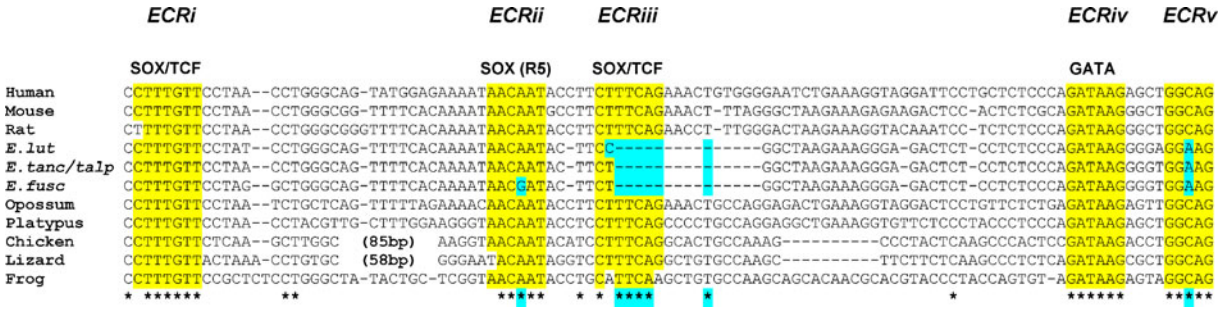


**Fig. 2** Sequence alignment of the *Ellobius ECRs* with those of other vertebrates. *ECRi* to *ECRv* (*highlighted in yellow*) are five highly conserved modules which were described in Bagheri-Fam et al. (2010). Transcription factors predicted to bind to these modules and the SOX site R5 are shown (Sekido and Lovell-Badge 2008; Bagheri-Fam et al. 2010). Sequence variations in *Ellobius* of nucleotides conserved across vertebrates (*asterisks*) are *highlighted in cyan*. Note that the highly conserved module *ECRiii* is absent in all *Ellobius* species analyzed due to a 14-bp deletion

**Table 1** DNA sequence identities (percent) calculated between the different mammalian *ECRs*

| Percent | *E. tanc* | *E. fus* | Mouse | Rat | Human |
|---|---|---|---|---|---|
| *E. lut* | 97 | | 75 (86[a]) | 72 (83[a]) | 66 (76[a]) |
| | *E. tanc* | 97 | 77 (89[a]) | 75 (86[a]) | 68 (78[a]) |
| | | *E. fus* | 75 (86[a]) | 72 (83[a]) | 65 (75[a]) |
| | | | Mouse | 88 | 81 |
| | | | | Rat | 83 |
| | | | | | Human |

sites, we used the online program MatInspector (www.genomatix.de) with core and matrix similarity set to 1.00 and optimized, respectively. As mentioned above, Δ14 removes most of *ECRiii* in all *Ellobius* species analyzed, and *E. lutescens* contains an additional T to C change (Fig. 2). Due to these sequence changes, MatInspector predicted the generation of new transcription factor binding sites in the *ECRs* of *E. lutescens* and *E. fuscocapillus*, namely an E-twenty-six (ETS) site in *E. lutescens* and a binding site for Heat transcription factors in *E. fuscocapillus* (Fig. 3, marked in red). The single nucleotide change (A to G) in module *ECRii* of *E. fuscocapillus* leads to loss of the predicted SOX site R5 (Fig. 3, marked in blue), while new predicted binding sites are generated for MYBL and HZIP (Fig. 3, marked in red). No transcription factor binding sites were predicted for module *ECRv* (data not shown).

## Δ14 increases basal activity and SF1-mediated activation of mouse *TESCO* in HEK293T cells

Previously, we have shown that in the presence of SF1, both SRY and SOX9 can synergistically activate mouse *TESCO* (Sekido and Lovell-Badge 2008). Since Δ14 removes a highly conserved potential SOX/TCF binding site (Fig. 2), we speculated that Δ14 might alter SRY and/or SOX9-mediated activation of mouse *TESCO*. To test this, we introduced Δ14 into mouse *TESCO* (*TESCO-Δ14*) by site-directed mutagenesis and cloned both *TESCO* and *TESCO*-Δ14 into a luciferase reporter vector. Human *SF1*, *SRY* and *SOX9* expression plasmids were co-transfected with either the *TESCO* or *TESCO-Δ14* reporter construct into the human embryonic kidney 293 (HEK293T) cell line

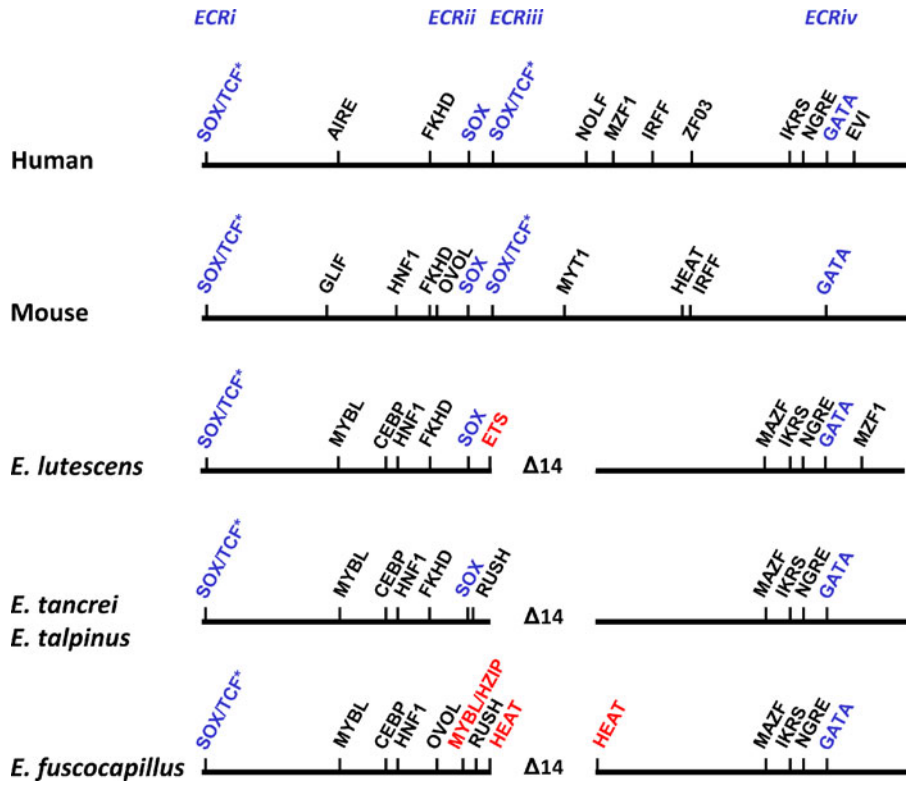


**Fig. 3** Putative transcription factor binding sites within the *ECRs* of human, mouse, *Ellobius lutescens*, *Ellobius tancrei* and *Ellobius fuscocapillus*. Transcription factor binding sites were predicted by the online program MatInspector (www.genomatix.de) with core and matrix similarity set to 1.00 and optimized, respectively. Transcription factor binding sites (1) marked by *asterisks* were identified by visual inspection (Bagheri-Fam et al. 2010), (2) *highlighted in blue* are located within the highly conserved modules *ECRi* to *ECRiv* and (3) *highlighted in red* are generated in *Ellobius* due to sequence changes in the highly conserved modules *ECRii* and *ECRiii*. Δ14 represents the 14-bp deletion found in all *Ellobius* species analyzed

($n$=5). We found that SF1 alone and SF1 together with SRY activated *TESCO-Δ14* significantly higher (~1.4-fold) than wild-type *TESCO* (Fig. 4a). In contrast, SOX9 together with SF1 activated *TESCO* and *TESCO-Δ14* to a similar extent (Fig. 4a). We also noted that basal activity of *TESCO-Δ14* was significantly higher (~2.5-fold) than that of wild-type *TESCO* in HEK293T cells (Fig. 4b). Taken together, these data indicate that Δ14 leads to increased activity of *TESCO*.

## Discussion

Through a PCR-based approach using degenerate primers, we were able to clone 1.1 kb of *E. lutescens TESCO* (including the entire *ECR*) which shares 75% sequence identity with mouse *TESCO*. This shows that *TESCO* is present in this species with high sequence conservation despite the loss of *Sry*. We did not expect a complete loss of *TESCO* in *E. lutescens* since *TESCO* is controlled by additional important transcription factors including SF1, SOX9 (SOX9 autoregulation) and FOXL2 (SOX9 repression) (Sekido and Lovell-Badge 2008; Uhlenhaut et al. 2009). Moreover, *TESCO* sequences are conserved in non-mammalian species including chicken, lizard and frog (Bagheri-Fam et al. 2010) which all lack *Sry* indicating that *TESCO* might be important for testicular *Sox9* expression independent of the sex-determining switch mechanism. It is thus possible that *Sox9* expression in *E. lutescens* is still regulated via *TESCO* by factors such as SOX9, SF1 and FOXL2 and potentially also by the new sex-determining switch gene which remains to be identified.

We could also clone the *ECR* of *E. tancrei* (which also lacks *Sry*) and of the *Sry*-positive species *E. fuscocapillus* spanning all evolutionarily conserved modules (*ECRi-ECRv*) which allowed us to compare the *Ellobius ECR* sequences with each other and to other vertebrate *ECRs*. All three *Ellobius* species carry a 14-bp deletion (Δ14) removing module *ECRiii* and additional 3′-flanking sequences. As a result, a putative SOX/TCF site is removed in all three *Ellobius* species, and new transcription factor binding sites are

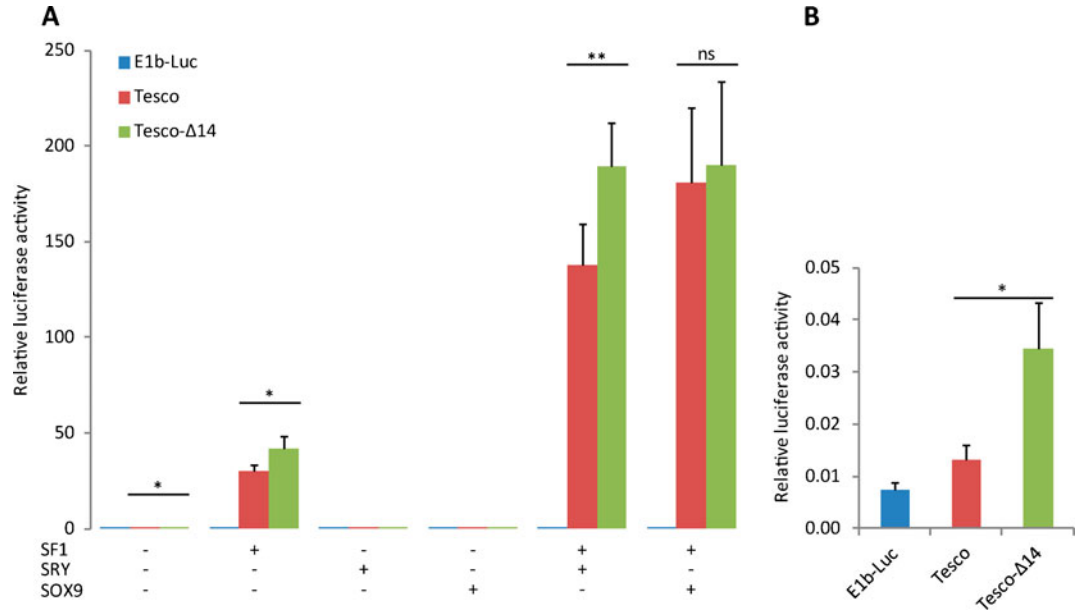


**Fig. 4** Δ14 increases SF1 and SF1-SRY-mediated activation and basal activity of mouse *TESCO* in HEK293T cells. **a** HEK293T cells were transiently co-transfected with E1b promoter-driven luciferase reporter constructs containing either intact *TESCO* or *TESCO* containing the 14-bp deletion found in *Ellobius* (*TESCO-Δ14*). The empty E1b-Luc vector was used as a negative control. Expression plasmids bearing human *SF1*, *SRY* and *SOX9* were also co-transfected into the cells. **b** HEK293T cells were transiently co-transfected with E1b promoter-driven luciferase reporter constructs containing either intact *TESCO* or *TESCO* containing the 14-bp deletion found in *Ellobius* (*TESCO-Δ14*). The empty E1b-Luc vector was used as a negative control. Means of normalised luciferase readings and standard error of the mean from five independent assays are shown. Paired $t$ tests were performed for statistical analysis. *$P$<0.05; **$P$<0.01

created, an ETS binding site in *E. lutescens* and a Heat protein binding site in *E. fuscocapillus*. The only sequence difference between the *Sry*-positive species *E. fuscocapillus* and the two *Sry*-negative *Ellobius* species within the highly conserved *ECR* modules is the A to G change in module *ECRiii* predicted to abolish SRY binding to the bona fide target site R5 (Sekido and Lovell-Badge 2008).

The presence of Δ14 in all *Ellobius* species is an intriguing finding since it shows that this deletion occurred before the speciation of *Ellobius* and thus before the loss of *Sry* (Fig. 5). So, could there be a link between Δ14 and the evolution of a *Sry*-independent sex-determining mechanism in the genus *Ellobius*? Our in vitro data show that Δ14 increased both basal activity and SF1-mediated activation of mouse *TESCO*. One potential explanation for this observation is that Δ14 removes a predicted TCF binding site which might mediate repression of *TESCO*. In the XY mouse gonad, over-expression of beta-catenin (which associates with TCF transcription factors to regulate target genes) leads to loss of SOX9 expression and male to female gonadal sex reversal (Maatouk et al. 2008) possibly by reducing SF1 binding to *TESCO* (Bernard et al. 2011). *Sox9* expression is initiated by SF1 in both sexes and is then up-regulated and maintained in XY gonads by SRY and SOX9, respectively, eventually leading to testis development (Sekido and Lovell-Badge 2008). Based on our functional assay, one could speculate that Δ14 might have raised *Sox9* expression in the gonads of the common ancestor of the *Ellobius* species above a threshold level allowing SOX9 to maintain its own expression. This would lead to female-to-male sex reversal in XX individuals and thus to a destabilization of the XY/XX sex-determining mechanism. Moreover, in XY individuals, the expression of *Sox9* and thus male development would become less dependent on or even independent of *Sry* (Fig. 5). This effect could be exacerbated through the inhibitory action of SOX9 on *Sry* transcription (Chaboissier et al. 2004; Barrionuevo et al. 2006). In support for such a scenario, XX transgenic mice over-expressing SOX9 develop as infertile males demonstrating that SOX9 is sufficient to induce maleness in the absence of *Sry* (Vidal et al. 2001). Evolutionary pressure for survival of such a *proto-Ellobius* species with predominantly male individuals could have resulted in speciation during which *E. lutescens/E. tancrei* and *E. fuscocapillus* independently stabilized their sex determination mechanisms, utilizing *Sry*-independent and *Sry*-dependent approaches, respectively (Fig. 5). In the former case (*E. lutescens/E. tancrei*), a female sex-determining gene might have evolved to down-regulate *Sox9* expression specifically in female gonads. In the latter case, *E. fuscocapillus* could have prevented

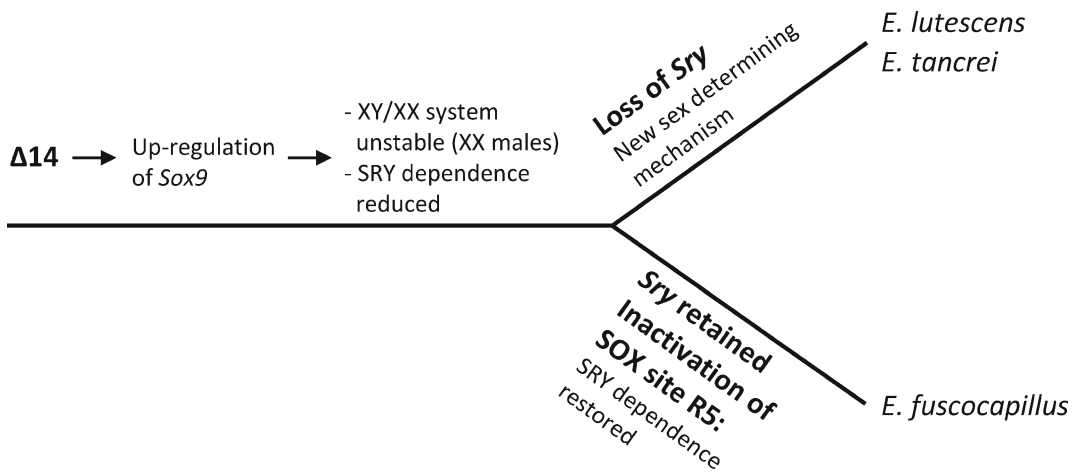


**Fig. 5** Putative model for the evolution of a *Sry*-independent sex-determining mechanism in *Ellobius lutescens* and *Ellobius tancrei*. A 14-bp deletion (Δ14) occurred in the common ancestor of *E. lutescens*, *E. tancrei* and *Ellobius fuscocapillus*. Δ14 might have increased *Sox9* expression in XX gonads thereby destabilizing the sex-determining mechanism in *Ellobius*. Subsequent evolutionary pressure for survival of this species could have resulted in speciation during which *E. fuscocapillus* and the common ancestor of *E. lutescens* and *E. tancrei* independently stabilized their sex determination mechanisms, utilizing *Sry*-dependent and *Sry*-independent approaches, respectively

the loss of *Sry* by restoring *Sox9* expression to normal levels in XX and XY gonads such as by loss of the important SRY/SOX binding site R5 (Figs. 2 and 3) thereby weakening *TESCO* activity. In such a scenario, the dependence of *Sox9* expression and male development on *Sry* would be restored (Fig. 5).

There are various possible new sex-determining mechanisms to replace *Sry* in *Ellobius*. For example, in the *Sry*-negative *Tokudaia* species, additional copies of *Cbx2* might trigger male sex determination (Kuroiwa et al. 2011). A potential link between a mutation in the testis-specific enhancer of *Sox9* and the evolution of a *Sry*-independent sex-determining mechanism is an intriguing hypothesis which remains to be tested.

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