REPORT

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SOHLH1 and SOHLH2 directly down-regulate STIMULATED BY RETINOIC ACID 8 (STRA8) expression

MG Desimio, F Campolo, S Dolci, M De Felici, and D Farini*

Department of Biomedicine; Section of Histology and Embryology; University of "Tor Vergata"; Rome, Italy

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Abbreviations: STRA8, Stimulated by Retinoic Acid 8; EC, Embyonal Carcinoma; RA, retinoic acid; PGC, primordial germ cell; RARE, Retinoic Acid Response Element; TF, transcription factor; ChIP, chromatin immunoprecipitation; dpc, days post coitum; dpp, days postpartum.

As the name implies, *Stimulated by Retinoic Acid* 8 is an early retinoic acid (RA) responsive gene pivotal for the beginning of meiosis in female and male germ cells. Its expression is strictly time-dependent and cell-specific (premeiotic germ cells) and likely requires a complex mechanism of regulation. In this study, we demonstrate a direct negative control of SOHLH1 and SOHLH2, 2 germ cell specific bHLH transcription factors, on *Stra8* expression. We observed a negative correlation between STRA8 and SOHLH1 expression in prepuberal differentiating mouse KIT^{+} spermatogonia and found that SOHLH1 and SOHLH2 were able to directly and cooperatively repress STRA8 expression in cell lines in vitro through binding to its promoter. We also identified 2 canonical E-Box motives in the *Stra8* promoter that mediated the negative regulation of SOHLH1 and SOHLH2 on these gene both in the cell lines and KIT^{+} spermatogonia. We hypothesize that this novel negative activity of SOHLH1and SOHLH2 in male cooperates with that of other transcription factors to coordinate spermatogonia differentiation and the RA-induced meiosis and in female ensures STRA8 down-regulation at mid-end stages of meiotic prophase I.

Introduction

Stimulated by Retinoic Acid Gene 8 (STRA8) protein has been proposed as the molecular effector of Retinoic Acid (RA) involved in meiosis-inductive activity. The mouse *Stra8* gene, originally identified in Embryonal Stem (ES) cells and germ cell-derived Embryonal Carcinoma (EC) cells after RA treatment,[1,2] is expressed at relatively high levels in male and female premeiotic germ cells. In female embryos lacking Stra8, the specification and the initial development of the primordial germ cells (PGCs), the precursor of the adult gametes, appear normal, but they fail to undergo premeiotic DNA replication and meiotic chromosome condensation.[3,4] In prenatal spermatogonia of *Stra8* deficient mice, the premeiotic DNA replication is conserved and spermatocytes are able to partly condense chromosomes and initiate meiotic recombination;[5,6] spermatocytes fail, however, to regularly continue over the leptotene stage of meiotic prophase I.[5]

Although these studies support the pivotal role of Stra8 in gametogenesis, the molecular function/s of the protein remain unknown. First described as a cytoplasmic protein,[2] it has been recently demonstrated in our laboratory, that it actually shuttles between the nucleus and cytoplasm.[7] It was also shown that STRA8 can bind to DNA and possesses a transcriptional activation domain in its C-terminal region.[7,8]

Although in several of developmental processes the basic molecular mechanisms underlying the RA action have been identified, its action in promoting entry into meiosis through *Stra8* induction has yet to be clarified. Actually it is known that in responding cells, RA usually serves as a ligand for 2 families of nuclear receptors, the RA receptors (RARα, β, or γ) and the retinoid X receptors (RXRα, β, or γ). RAR–RXR heterodimers bind to specific genomic DNA sequences, known as Retinoic Acid Response Elements (RARE), and regulate gene expression (for a review see refs. 9–10 and references here in). A number of studies showing that specific antagonists or agonists of the RARs were able to influence the onset of meiosis and the expression of *Stra8* in cultured germ cells, suggested a canonical mode of RA action on the germ cell meiotic entry (for a review see ref. 11). This notion was initially also supported by the report that the *Stra8* promoter contains 2 RARE, corresponding to DR2 and IR5 type, located in a 400 bp region upstream of the *Stra8*

*Correspondence to: D Farini; Email: donatella.farini@uniroma2.it
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transcriptional start site (TSS).[12] However, the importance of such elements for *Stra8* regulation remains controversial. In particular, it was reported that in the fetal ovary of *Raldh2*$^{-/-}$ mice, that lack RA synthesis, *Stra8* expression occurs normally.[13] In line with this, Le Bouffant et al.[14] showed that in embryonic mouse ovary the ablation of the homeobox *Msh-like* (*Msx*) genes 1 and 2 resulted in downregulation of *Stra8*. Moreover, *Doublesex and mab-3 related transcription factor 1* (*Dmrt1*) and 6 (*Dmrt6*) appear to play a crucial role in regulating meiosis and *Stra8* expression.[15-18] In particular, DMRT1 acts in a opposite sex-dependent way. Both in fetal female germ cells and postnatal male germ cells, DMRT1 is able to bind to the *Stra8* promoter.[15-18] While in female germ cells the ablation of *Dmrt1* results in lack of *Stra8* expression,[16] in male germ cells it caused uncontrolled meiotic entry.[15] In these latter, DMRT1 regulates the responsiveness to RA repressing directly the transcription of *Stra8* and delaying the enter into meiosis until spermatogonia are not completely competent.[15]

Stra8 expression appears also under the control of epigenetic events. Feng et al.[19] have recently reported that in the female mouse germ cells, NOTCH signaling is probably necessary for maintaining the epigenetic state of *Stra8* in a way suitable for RA stimulation. In F9 EC cells, Trichostatin-A (TSA), an inhibitor of class I/II histone deacetylase (HDAC), was found to mimic and synergize the inductive effect of RA on *Stra8* expression.[20] In ES cells, the expression of *Stra8* was shown to be regulated by CBP and p300, 2 histone acetyltransferase (HAT)-activity-containing proteins, while RA itself was reported to increase histone acetylation at the *Stra8* promoter.[21] In these cells, the most prominent binding sites for CTCFL (BORIS), a key coordinator factor of the 3-dimensional chromatin structure, are localized on the first intronic region of *Stra8* and when CTCFL was overexpressed, *Stra8* was induced.[22]

Finally, recent results suggest that, like other genes transcriptionally activated by RA at early times, in female germ cells *Stra8* resides in bivalent chromatin region and the Polycomb repressive complex 1 (PRC1) functions as a gatekeeper that control the responsiveness of premeiotic germ cells to the RA signaling.[23]

SOHLH1 and SOHLH2, 2 testis and ovary-specific bHLH transcription factors (TFs), essential for both spermatogenesis[24-27] and oogenesis,[28,29] might also play a role in the regulation of *Stra8* expression. In the mouse, SOHLH1 is expressed in A_{al}, A1–A4, Intermediate spermatogonia and at lower level in B spermatogonia[24,27,30] and is up-regulated by RA treatment.[30] SOHLH2 is expressed in all A spermatogonia (A_s–A_{al} and A1–A4) and absent in B spermatogonia.[25-27,30] Moreover, Sohlh1- and Sohlh2-knockout mice share common phenotypes, which in homozygosity leads to sterility both in testes and ovary.[24-29] STRA8 is upregulated either in female or in male germ cells of both mutants,[26-29] suggesting that SOHLH1 and SOHLH2 might exert an inhibitory action on *Stra8* expression. Actually, here we report a novel repressive role of SOHLH1 and SOHLH2 on *Stra8* expression through direct binding to 2 E-box motives in the -1,400 bp region of its promoter.

Results

Immunolocalization of STRA8 and SOHLH1 in postnatal mouse testis

It is well known that spermatogonia differentiation is a RA-dependent event accompanied by the acquisition of *Kit* expression[31] and that both undifferentiated (KIT$^-$) and differentiating (KIT$^+$) spermatogonia constitute an heterogeneous cell population. Previous results showed that populations of KIT$^+$ spermatogonia express SOHLH1[27,30] and STRA8,[32] while KIT$^-$ spermatogonia express primarily SOHLH2[27,30] but not STRA8. In order to determine in single spermatogonia whether there is a correlation between the expression of SOHLH1 and STRA8, we immunoocalized these proteins in testes from 7 dpp mice. We observed that STRA8 and SOHLH1 were expressed in a subset of both KIT$^+$ and KIT$^-$ spermatogonia and that the majority (about 70%) of the SOHLH1$^+$ cells were negative for STRA8 and vice versa (**Fig. 1**), raising the possibility of a functional negative relationship between the 2 proteins.

SOHLH1 and SOHLH2 down-regulate Stra8 expression

Sohlh1 and Sohlh2 are able to form homo-and heterodimers and bind E-Box motives in DNA of their target genes.[26-28,30] They have been mainly described as positive transcriptional regulators and their role as transcriptional repressors is not well characterized.[26-28] To verify whether SOHLH1 and SOHLH2 might negatively regulate *Stra8* expression, we transfected pcDNA3-*Sohlh1*, pcDNA3-*Sohlh2* or both expression vectors in the germ cells-derived P19 Embryonal Carcinoma (EC) cells, that express *Stra8* upon RA stimulation. We observed that the overexpression of either *Sohlh1* or *Sohlh2* significantly down-regulated *Stra8* levels stimulated by RA both at RNA and protein level while they did not affect the expression of the co-transfected control *Egfp* reporter (**Fig. 2B**). Moreover *Stra8* downregulation resulted higher following the co-expression of either transcription factors (TFs) (**Fig. 2A, B**). As expected,[30] overexpression of SOHLH1 and/or SOHLH2 in P19EC cells stimulated endogenous KIT expression (**Fig. S1**).

SOHLH1 and SOHLH2 down-regulate the *Stra8* promoter activity through E-Box DNA binding motif

Sequence analysis of the −1,400 bp regulatory region of the mouse *Stra8* gene for the presence of canonical E-Box motif (namely CANNTG), revealed the presence of 3 putative E-Box sites at positions −558 bp (CATCTG;) (Ebox-3, proximal), −841 bp (CAAGTG) (Ebox-2, middle) and −853 bp (CAGCTG) (Ebox-1, distal), relative to the transcriptional start site (TSS) (**Fig. 3**). The −1,400 bp region, located upstream from TSS (+1), includes the TATA promoter sequence and it is known to drive the expression of *Stra8* in the testis[32-33] and fetal ovary.[35]

To verify whether SOHLH1 and SOHLH2 were able to modulate the promoter activity of *Stra8*, we generated 2 expression vectors in which the −1,400 bp and −400 bp regulatory regions drive the expression of the *Luciferase* genes

(pGL3−1,400-*Stra8*-Luc and pGL3-400−*Stra8*-Luc vectors) and *Egfp* (−1,400-*Stra8-Egfp*; -400-*Stra8-Egfp* vectors). These plasmids were transiently transfected in P19 EC cells (not shown) and HEK293T (used primarily in such experiments for their higher transfection efficiency) along with *Sohlh1* or *Sohlh2* or both expression vectors or with an empty vector as a control. *Renilla* luciferase was used as an internal control for normalization of transfection efficiency. Lysates were obtained 48 hr after transfection and after 24 hr from the addition of 1 μM RA. The results in **Fig. 4A** (insert) showed that the plasmid construct containing the −1,400 bp-*Stra8* regulatory region upstream the *Luciferase* gene [(containing the DR4 (according to Raverdeau et al.,[36] DR2 and IR5 RARE sequences) was responsive to RA treatment. When the HEK293T or P19 EC cells (data not shown) were transfected with *Sohlh1* or *Sohlh2* or both plasmids, *Stra8* directed luciferase activity was significantly inhibited. Although either TFs showed a significant inhibitory activity, we found that co-transfection of both *Sohlh1* and *Sohlh2* maximally inhibited *Stra8*-mediated luciferase activity. We found that the −400 bp region of the *Stra8* promoter that contains the TATA box and the 2 putative RARE elements (DR2 and IR5) but not the E-Boxes, was not responsive to RA stimulation in HEK293T or P19 EC (**Fig. 4B**, insert). As expected, luciferase activity was not influenced by *Sohlh1* or *Sohlh2* or both plasmids co-transfection following RA stimulation. We also used *Egfp* as a reporter present in 2 *Stra8* promoter plasmids (p-1,400-*Stra8-Egfp,* p−400-*Stra8-Egfp*). Co-transfection of each plasmid with *Myc-Sohlh1* or *Myc-Sohlh2* or both in HEK293T cells was followed by stimulation with RA for 24 hr and the levels of EGFP evaluated by Western blot analysis. To normalize for the transfection efficiency, we introduced in all samples a vector expressing a Myc-Tyr fragment, a construct that was previously validated to be constantly expressed and inert.[30,37] As shown in **Figure 4C,** we found that SOHLH1 or SOHLH2 were able to significantly inhibit EGFP expression when compared to mock-transfected cells. Also in this case, EGFP down-regulation was stronger when both TFs were simultaneously introduced into the cells (**Fig. 4C**). As for the luciferase reporter assays, EGFP levels were not affected by the SOHLHs when the −400 bp region of *Stra8* promoter was transfected (**Fig. 4D**). To understand if the E-boxes present in the *Stra8* promoter mediate SOHLHs action, we constructed pGL3-CMV-Luc vectors containing all the 3 E-boxes (E1-E3; -922-521 bp), the first and second (E1-E2; -922-805 bp) and only the third (E3; -681-521 bp) (**Fig. 5A**, upper panel) and assayed their transcriptional activities in HEK293T cells in the presence of *Sohlh1* or *Sohlh2* or both expressing plasmids. The transcriptional assays showed that the region containing the 3 E-Boxes functioned as enhancer element and promoted higher basal transcriptional activity (**Fig. 5A**). When *Sohlh1* and *Sohlh2* were transfected alone or in combination, the luciferase activity was significantly inhibited when the E1 and E2 sequences were present, while E3 was dispensable (**Fig. 5A**). To determine whether the regulatory

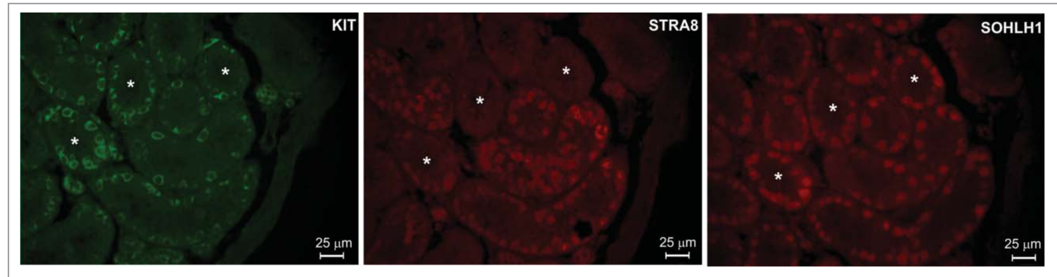

Figure 1. KIT$^+$ spermatogonia are an heterogeneous populations of cells concerning STRA8 and SOHLH1 expression. Immunodetection in adjacent sections of KIT, STRA8 and SOHLH1 in histological sections from 7 ddp mouse testis. Asterisks indicate sections in which SOHLH1$^+$ cells are STRA8$^-$.

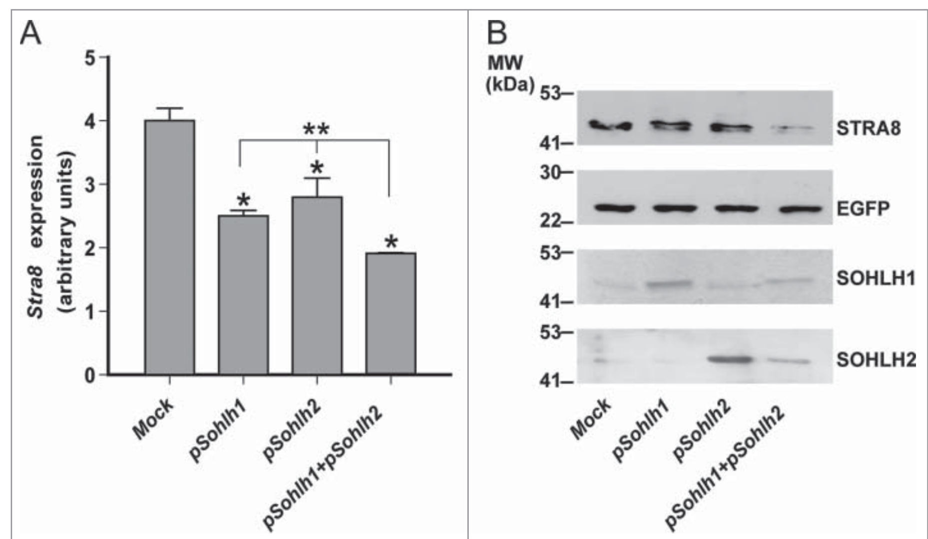

Figure 2. Overexpression of SOHLH1 and/or SOHLH2 inhibits Retinoic Acid (RA)-stimulated *Stra8* expression in P19EC cells. (**A**) qRT-PCR analysis of *Stra8* expression in P19EC cells electropored with the indicated plasmids and treated, after 4 hr of culture, with 1 μM RA for additional 24 hr. Each qRT-PCR was carried out in triplicate. Bars indicate the mean ± SEM, n = 3 different transfection. *$P \leq$ 0.01 respect to the Mock; **$P \leq$ 0.01 for *Stra8* expression levels in *Sohlh1* plus *Sohlh2* co-transfected P19EC cells versus single transfectants. (**B**) Western blot analysis of STRA8 levels in P19EC cells electropored with limiting concentration of p*Egfp* expressing vector and pcDNA3-*Sohlh1*, pcDNa3-*Sohlh2*, alone or together and treated as above.

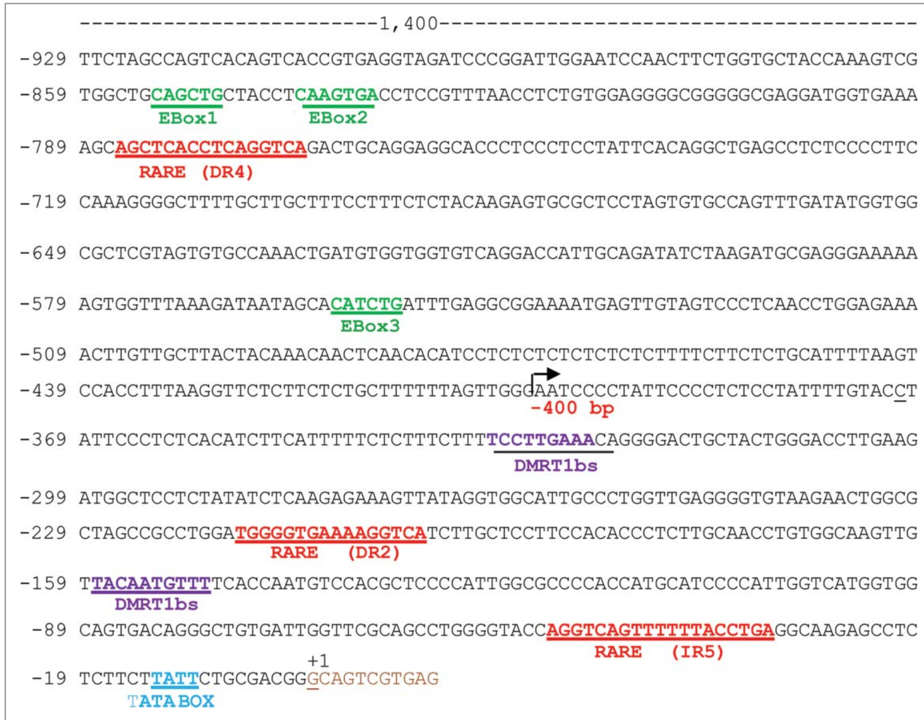

Figure 3. Partial schematic representation of the −1,400 *Stra8* promoter region. +1 indicates the start of transcription. The TATA-box like sequence (blue), the putative binding sites (RARE, red) for retinoic acid receptor, for DMRT1 (purple) and the 3 E-Boxes (green) are indicated. It is also indicated the begin of the −400-*Stra8*-promoter construct ( ).

action of SOHLHs on the *Stra8* promoter was mediated by the E-Boxes, we mutated the 2 internal bases in E1, E2 and E3 motives of the pGL3−1,400-*Stra8*-Luc construct (CAcgTG, CAgaTG and CActTG respectively) and expressed them in reporter assays with *Sohlh1* and *Sohlh2* (**Fig. 5B**). Mutation of either E1 or E2 strongly reduced the inhibitory effect of both SOHLH1 and SOHLH2, while the E3-mutated *consensus* did not influence the inhibitory activity of the TFs (**Fig. 5B**). Comparable results with an E1/E2/E3 triple-mutant promoter were obtained (**Fig. 5B**), thus suggesting that the adjacent E1 and E2 E-boxes were the most important site of SOHLH1 and SOHLH2 binding.

SOHLH1 binds in vivo the upstream regulatory region of *Stra8* gene in prepuberal spermatogonia

In order to verify whether that SOHLH1 was able to bind the E-Boxes-containing-*Stra8* regulatory region, we performed ChIP assays by immunoprecipitating SOHLH1 from formaldehyde cross-linked chromatin obtained from 7 dpp spermatogonia. We designed primer pair that specifically amplify the genomic fragments containing the first 2 E-box sequences (region A) and primer pair that amplify an intronic region in which E-Boxes were not included (region B) (**Fig. 6A**). Using PCR, we found that only the region containing the E1 and E2 E-boxes sequences was significantly enriched in SOHLH1-immunoprecipitated DNA, while region B, which did not contain E-boxes, was not amplified from SOHLH1-immunoprecipitated chromatin (**Fig. 6B**). We used the mouse NIH3T3 cell line that did not express SOHLHs as a negative control and as expected, no amplicon was present in the immunoprecipitation when using both primer pairs (**Fig. 6C**). In addition, we repeated ChIP experiments on chromatin from both KIT^{-} and KIT^{+} spermatogonia. The results reported in **Figure 6D** showed that a significant binding of SOHLH1 was achieved mostly to the chromatin region A obtained from KIT^{+} spermatogonia.

Discussion

A number of studies indicate that Stimulated by Retinoic Acid 8 (STRA8) is crucial for the beginning of meiosis both in embryonic female and postnatal male germ cells.[3-6] However, as reported in the Introduction, the regulation of the *Stra8* gene by RA, at least in the embryonal ovary, is controversial[38] while the role of the encoded protein remains a mystery.

A key event of spermatogenesis, is the transition from spermatogonial mitotic proliferation and differentiation to meiotic entry by spermatocyte. This complex differentiation program is under the control, among others, of RA (for a review see ref. 39). Although several genes that control the early stage of spermatogenesis have been identified, how spermatogonia differentiation is regulated and coordinated with the beginning of meiosis remains poorly understood. (for a review see ref. 40). Several transcription factors have been shown to control spermatogonia maturation. Among these, DMRTs and SOHLHs play a pivotal role. DMRT1 and DMRT6 cooperate in repressing or activating genes involved in spermatogonia differentiation and mitotic/meiotic shift.[15,17] Downstream DMRTs are SOHLH1 and SOHLH2 that suppress genes involved in the maintenance of spermatogonial stem cells and induce genes important for spermatogonia differentiation.[24-27,30] In the present study, we described in details a novel negative regulatory role exerted by SOHLH1 and SOHLH2 on the *Stra8* gene expression.

The observations that DMRT1 regulated spermatogonial commitment to meiosis inducing *Sohlh1* and suppressing *Stra8* expression[15] and that *Stra8* was up-regulated either in female and male germ cells of *Sohlh1* and *Sohlh2* knockout mice,[26-29] prompted us to investigate whether SOHLH1 and SOHLH2 actually may directly suppress *Stra8* expression. A first positive confirmation of this hypothesis came from the

immunolocalization of the SOHLH1 and STRA8 proteins in single prepuberal spermatogonia. We observed that in some seminiferous tubules con-Kit+ spermatogonia were both STRA8 positive or STRA8 negative. Moreover, in other tubules, STRA8 was clearly present in KIT− spermatogonia, probably correspond ing to the B spermatogonia or preleptotene spermatocyte in which *Kit* starts to be downregulated.[41] The heterogeneity of the spermatogonia population was even more evident observing the contemporary expression of KIT, STRA8 and SOHLH1 that are all markers of differentiating spermatogonia regulated by RA.[30,32,41] In particular, we noticed that, as a rule, the expression of SOHLH1 and STRA8 was mutually exclusive. This observation parallels the distribution of SOHLH1 in the adult testis reported in previous studies in which SOHLH1 in KIT+ differentiating spermatogonia was shown to gradually disappears in positive intermediate and B spermatogonia[27] that are STRA8 positive.[32,42]

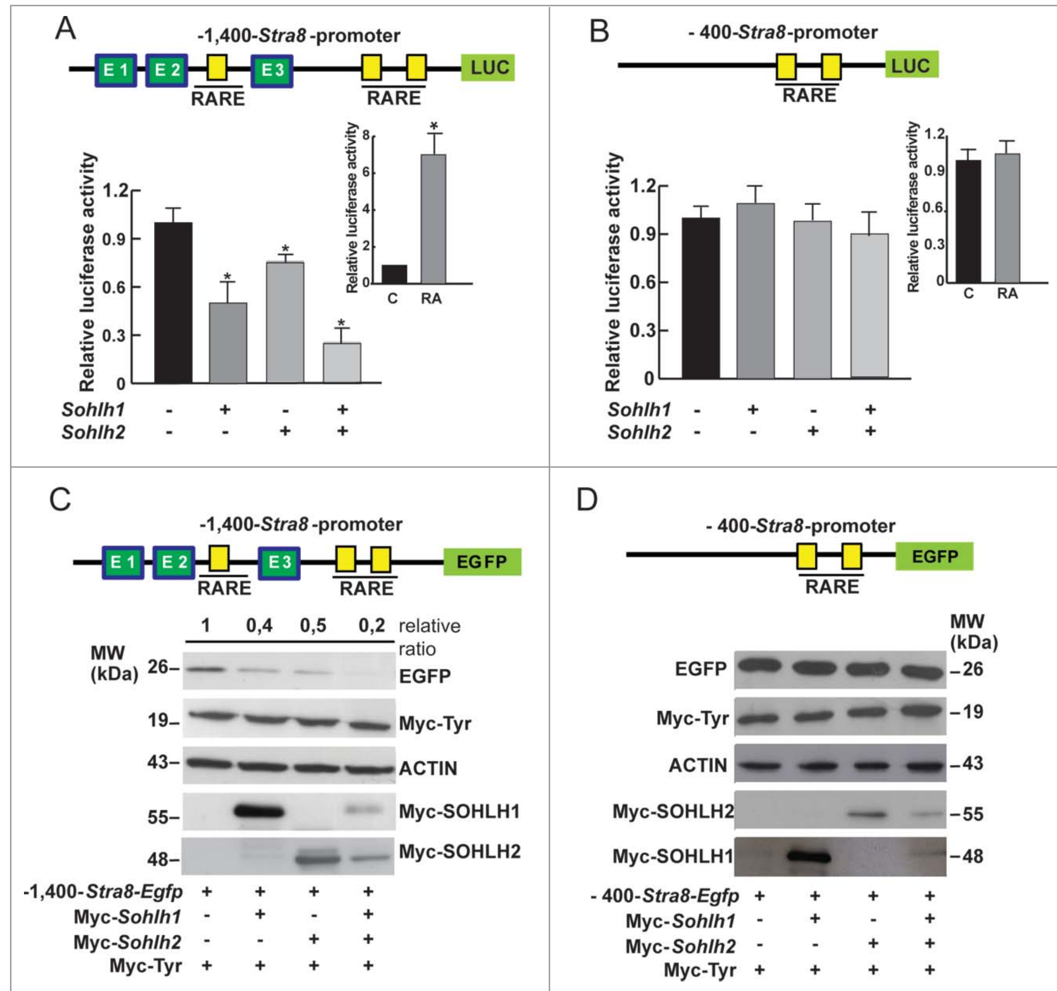

Figure 4. SOHLH1 and SOHLH2 repress the *Stra8* promoter activity. (**A**) HEK293T cells were co-transfected with 200 ng of reporter pGL3-1,400-*Stra8-Luc* (upper panel) and with different amounts of pcDNA3-*Sohlh1*- and/or pcDNA3-*Sohlh2*-expressing vectors as indicated in the Materials and Methods. The cells were treated with 1 μM RA 24 hr after transfection. After 48 hr, cells were harvested and lysed, and luciferase activity were determined and normalized by *Renilla* luciferase. The results are shown as relative luciferase activity (± SEM) (**P* <0.01). The transfections were repeated 3 times. Insert shows the effect of RA treatment in mock- transfected cell . (**B**) *Sohlh1* and/or *Sohlh2*-expressing vectors were co-transfected in HEK293T cells with the deletion mutant pGL3-400-*Stra8-Luc* (upper panel) and the luciferase assay was performed as above (mean ± SEM from three different experiments). Relative luciferase activity in mock-transfected cells treated with RA is shown in the insert. (**C**) Western blot analysis of EGFP expression in HEK293T cells co-transfected with 500 ng of p1,400-*Stra8-Egfp* reporter, shown in the upper panel, and with *Myc-Sohlh1*- and/or *Myc-Sohlh2*-expressing vectors (500 ng or 250 ng). The *Myc-Tyr*-expressing plasmid was co-transfected in all cases to normalize the transfection efficiency. 1 μM RA was added to the medium 24 hr after transfection and the cells were lysed 48 hr after transfection. The relative ratio of the EGFP expression levels were estimated compared with the reporter expression levels without *Sohlh*s. (**D**) Western blot analysis was performed as above using the p400-*Stra8-Egfp* reporter shown in the upper panel.

Subsequent analyses in P19 Embryonal Carcinoma (EC) cell line responsive to RA and transfected with suitable probes, confirmed the repressive action of both SOHLH1 and SOHLH2 on the *Stra8* gene expression. Moreover, they indicated that both TFs exerted a cooperative direct inhibition of the gene by binding to 2 canonical E-Box motives within the −1,400 bp regulatory region of the *Stra8* promoter known to drive the expression of the gene both in testis [33,34] and fetal ovary.[35] The cooperation between SOHLH1 and SOHLH2 in repressing *Stra8* is not surprising since, as other bHLH transcription factors, they function in spermatogonia by forming homo-or heterodimers[27,30,43] and in this last configuration, they are more efficient, for example in stimulating *Kit* expression.[30] We speculate that the cooperation between SOHLH1 and SOHLH2 in repressing *Stra8* might ensure a more stringent negative regulation in those KIT+ not fully differentiated spermatogonia in which SOHLH2 is still present and SOHLH1 starts to be expressed.

The finding that in a ChIP assay, SOHLH1 bounds the E1 and E2 Box motives of the *Stra8* promoter in the chromatin obtained from KIT+ spermatogonia, strongly supports a

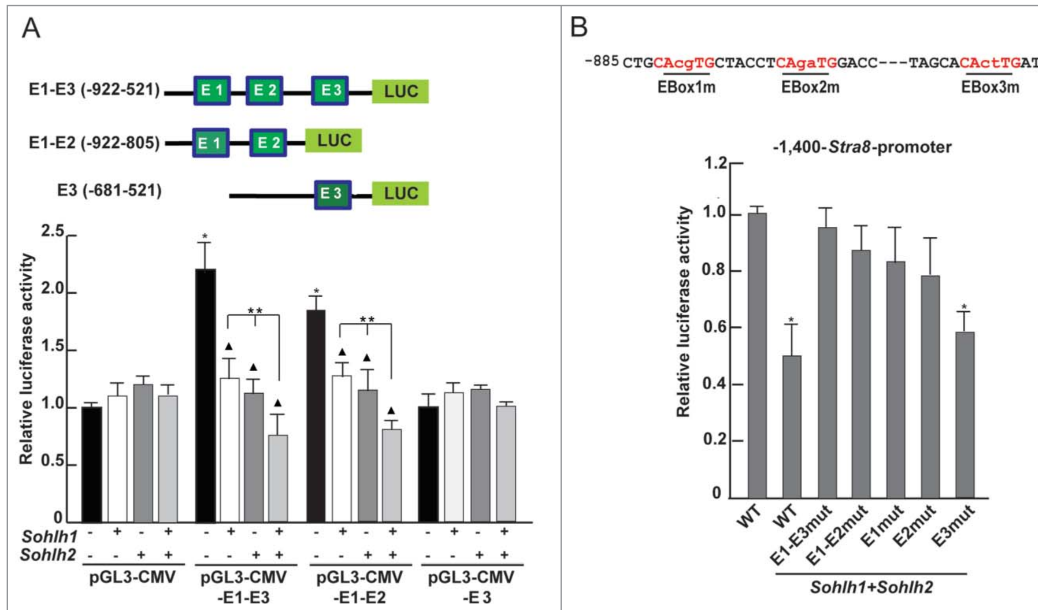

Figure 5. SOHLH1 and SOHLH2 down-regulate the *Stra8* promoter activity through E-Box binding motif. (**A**) Upper panel: schematic representation of the different portions of the *Stra8* promoter used in this reporter experiments that contain canonical E-box consensus sequences indicated in green. Lower panel**:** co-transfection of the different reporter constructs with Sohlh1- and/or Sohlh2-expressing vectors into HEK293T cells. Luciferase activities were measured 48 hr after transfection and normalized to the Renilla activity. Bars depict the means $\pm$ SEM of 3 experiments. *$P < 0.01$ respect to the pGLG3-CMV Mock; ▲ $P < 0.01$ respect to the Mock. **$P < 0.01$ in *Sohlh1* plus *Sohlh2* co-transfected cells vs. single transfectants. (**B**)**.** Upper panel**:** illustration of mutated E-Box sequences, in each of which the 2 internal bases were replaced as indicated. Lower panel: mutational analysis of the 3 E-Boxes in the *Stra8* promoter in the presence of overexpressed *Sohlh1* and Sohlh2. HEK293T cells were co-transfected with 200 ng of WT and mutant pGL3-1,400-*Stra8-Luc* reporter constructs and 400 ng of pcDNA3-*Sohlh1* and pcDNA3-*Sohlh2* and treated with 1 μM RA after 24 hr. Reporter assays were performed as above.

regulative role of this transcription factor on the *Stra8* gene expression in such cells.

Since STRA8 is pivotal for the beginning of meiotic prophase, it is not surprising that its expression must be finely regulated. In this contest, our data suggest that SOHLH1 and SOHLH2, directly repressing *Stra8* expression in the presence of RA, contribute together with DMRT1 and DMRT6 to ensure that meiosis starts only when spermatogonia reached the appropriate differentiation.

In the fetal ovary, in contrast to the testis, DMRT1 is a positive direct regulator of *Stra8* expression,[16] while 2 homeobox proteins, MSX1 and MSX2, appear necessary for the maintenance of *Stra8* level in the meiotic oocytes.[14] Moreover, the Polycomb repressive complex (PRC1) is important to maintain *Stra8* repressed until the female PGCs do not acquire the meiotic competence.[23] Interestingly, in these cells the *Stra8* promoter is characterized by a bivalent chromatin state, in which an activating (H3K4me3) and a repressive (H3K27me3) modification of the histones are contemporary present.[23] In the female germ line, SOHLH2, but not SOHLH1, was expressed as early as 12.5 dpc and remains expressed at least up to 15.5 dpc.[30] Since *Stra8* begins to be expressed around 12.5 dpc and is completely down-regulated 3–4 d later, a repressive action of SOHLH2 on *Stra8* during this window time can be excluded. This is, however, possible during later meiotic prophase I stages, since in *Sohlh1* and *Sohlh2*-knockout oocytes, *Stra8* is the only meiotic gene to be abnormally upregulated at perinatal stages.[26,28] At this stages, SOHLH1 has been demonstrated to transcriptionally stimulate through binding to canonical E-box sequences, the promoter activity of *Lhx8* and *Zp1*, 2 genes crucial for the progression of primordial follicles into primary follicles.[28]

Actually until now, SOHLH1 and SOHLH2 were known as positive regulators of gene expression both in male and female germ cells.[26-28,30] Here we show, for the first time, that by binding to E-Boxes, SOHLH1 and SOHLH2 are able to negatively modulate gene transcription. Such double activatory and inhibitory action is common to other members of the bHLH TF family. For example, myogenic regulatory factors, such as MyoD, Myf5, myogenin, and MRF4, all members of the same bHLH TF family, can either activate or repress the transcription of target genes.[44] The posttranslational modifications,[45] the epigenetic modification of chromatin, the ability to associate with both histone acetylase or deacetylase and recruitment of additional proteins to the transcriptional complex are all involved in the specific mode of action of these TFs (for a review see refs. 46, 47).

We do not known how SOHLH1 and SOHLH2 by binding to E-Boxes, are able to exert opposite effects in spermatogonia, negative on the *Stra8* promoter and positive on *Kit* promoter[30] and *Sohlh1* promoter itself.[43] In the last case, it is the ability of SOHLH1 and SOHLH2 to form a ternary complex with SP1 that binds a GC rich sequence adjacent to the E-Boxes, that results in strong promoter activation. Since the repressive action of SOHLHs occurs in the presence of elevated level of RA, interaction with the RAR/RXR and/or repressive epigenetic modification of the chromatin are possible and will be investigated in future studies.

Materials and Methods

Immunohistochemistry

For immunohistochemistry, serial 5 μm thick sections were obtained from testes of 7 dpp CD1 mice, fixed in buffered

formalin and paraffin embedded. Slides were dewaxed, rehydrated, and microwaved in 10 mM sodium citrate buffer, pH 6 for 10 min. After blocking with 10% goat serum, sections were incubated with preimmune serum and with rabbit polyclonal anti-KIT (1:100 AF1356, R&D System), anti-SOHLH1 (1:200 Ab41520, Abcam) and anti-STRA8 (1:400 Ab49405, Abcam) antibodies diluted in PBS/0.5% BSA at 4°C overnight. 1:500 goat anti-rabbit (Alexafluor 568, Thermo Fisher Scientific Inc.) were used as secondary antibodies for 1 hr incubation at RT. Samples were visualized under a Leica CTR600 microscope with a 40X objective.

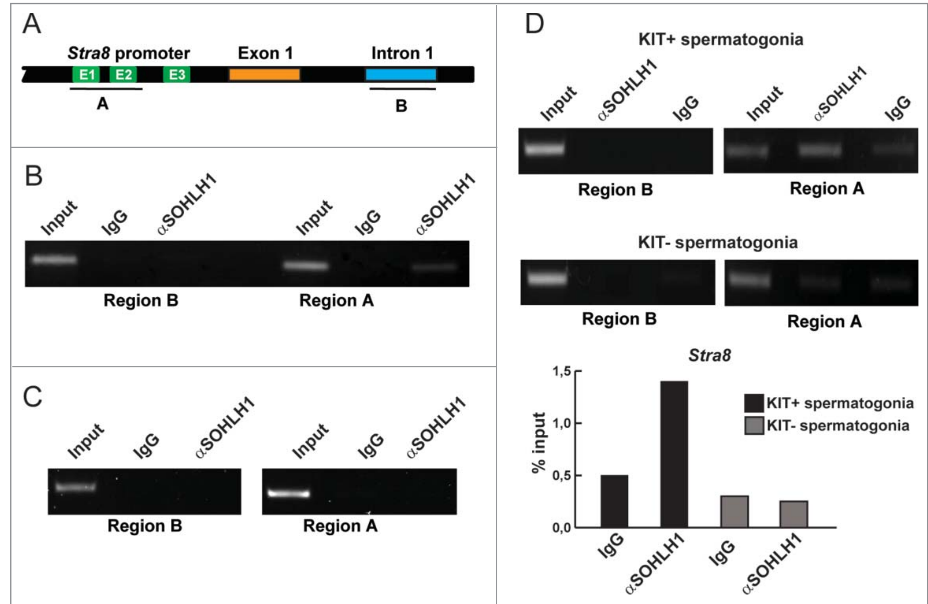

Figure 6. SOHLH1 binds to the *Stra8* promoter of mainly KIT+ differentiating spermatogonia. (**A**) *Stra8* regulatory region flanking exon 1 is displayed showing region A that contains the E-boxes (E1-E3) and region B in the first intron used as negative control. (**B**) Chromatin immunoprecipitation (ChIP) assay on spermatogonia prepared from 7 dpp mouse testes. The antibody against SOHLH1 precipitates genomic DNA of the *Stra8* promoter containing E-Boxes. Input was the PCR product from 2% of the DNA used before IP. (**C**) ChIP experiment on NIH3T3 cells used as a negative control. (**D**) ChIP assays on KIT+ and KIT− spermatogonia isolated from 7 dpp mouse testes. Lower panel: densitometric quantification of SOHLH1 binding (% input) from the above ChIP results was performed by determining the amount of SOHLH1-specific signal compared with input for KIT+ or KIT− spermatogonia.

DNA constructs

The coding region of mouse *Sohlh1* (GenBank NM_001001714) and *Sohlh2* (GenBank NM_028937) was amplified by RT-PCR from 1 microgram of total RNA obtained from 10 dpp mouse testis using primers 1–4 listed in **Table 1** and cloned in pcDNA3 vector. Plasmids expressing the fusion protein Myc-SOHLH1 and Myc–SOHLH2 were constructed subcloning the coding sequence of mouse *Sohlh1* and *Sohlh2* within the N-terminus of pCDNA3-N2Myc by using restriction enzymes EcoRI and XhoI for *Sohlh1* and BamHI and XhoI for *Sohlh2* (primers 1–2 and 3–4 respectively, **Table 1**). The −1,400-*Stra8-Egfp* plasmid was generously provided by Prof. JL. Tilly (University of Boston, USA). The −400-*Stra8-Egfp* vector was obtained subcloning the corresponding region (-400 bp +11 bp) amplified by PCR using the -1,400-*Stra8-Egfp* as template and primers 5–6 listed in **Table 1**. Plasmids used for luciferase reporter assays were obtained by subcloning the *Stra8*-promoter regions in pGL3-basic-vector (Promega) by using restriction enzymes XhoI/NcoI (pGL3-1,400-*Stra8*-Luc; pGL3-400-*Stra8*-Luc). The deletion mutants containing the E-Boxes E1-E3 (−922-521 bp), E1-E2 (−922-805 bp) and E3 (−652-

Table 1. Sequences of the primers used in this study

		Sequence 5′-3′
1	p-*Sohlh1*-for	AG**GAATTC**ATGGCGTCCGGTGGCCAC
2	p-*Sohlh1*-rev	AG**CTCGAG**TCAGGGGAAAAAGTCAGG
3	p-*Sohlh2*-for	AG**GGATCC**ATGGCCGACCGGATCAGC
4	p-*Sohlh2*-rev	AG**CTCGAG**TTAAAAGTAACTACTGTC
5	−400-*Stra8*- for	AG**CTCGAG**CTATTCCCTCTCACATCT
6	−400-*Stra8*- rev	AG**GAATTC**GCCCTGACGAGTCAGTCA
7	E1-luc for	AG**CTCGAG**CAGTCACCGTGAGGTAGATCCCGG
8	E3-luc rev	AG**CCATGG**GAGGGACTACAACTCATTTTCCGCC
9	E2-luc rev	AG**CCATGG**CCCGCCCCTCCACAGAGGTTA
10	E3-luc for	AG**CTCGAG**TGGCGCTCGTAGTGTGCCAA
11	E1mut	CTACCAAAGTCGTGGCTG*ca**att**g*CTACCTCAAGTGAC
12	E2mut	AACGGAGGT*ca**tct**g*AGGTAGCAGCTGCAGCCAC
13	E3 mut	TTTTCCGCCTCAAAT*ca**agt**g*TGCTATTATCTTTAAACCACTTTTTTC
14	*Stra8* for	GTTCCTGCGTGTTCCACAAG
15	*Stra8* rev	CACCCGAGGCTCAAGCTTC
16	*Gapdh* for	AACTTTGGCATTGTGGAAGG
17	*Gapdh* rev	CCGTGTTCCTACCCCCAATGTG

521 bp) were obtained amplifying the corresponding fragments by PCR by using 1,400-*Stra8-Egfp* as template and primers 7–10 as indicated in **Table 1** and cloning them in pGL3-CMV-vector (Promega). Mutation of E-Box binding sites in pGL3-1,400-*Stra8*-Luc were introduced by QuickChange Lightning Multi Site-Directed Mutagenesis Kit (#210513, Agilent Technologies) using primers designed with QuickChange Primer Design Program and listed in **Table 1**. 100 ng of pGL3-1,400-*Stra8*-Luc were used as template with 100 ng of mutagenic primers and the synthesis reaction was cycled as indicated in instruction manual. All cloned constructs and mutants were confirmed by DNA sequencing (BMR, University of Padova, Italy).

Cell transfection

Mouse P19 embryonic carcinoma cells (ECP19, ATCC CRL-1825) were grown on feeder-free, gelatine-coated plates in D-MEM (Life Technologies) supplemented with 2 mM glutamine, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1× nonessential amino acids (all from Sigma-Aldrich) and with 10% fetal bovine serum (FBS; Life Technologies). For STRA8 expression studies, plasmids were introduced into undifferentiated cells by means of a Nucleofector system (Amaxa Biosystems) according to the manufacturer's instructions. Briefly, 2 $\times 10^6$ cells were electroporated with 2 μg of pcDNA3-*Sohlh1* or pcDNA3-*Sohlh2* plasmid vectors or 1μg of each in the case of co-transfection. 200 ng of pEGFP-C1 (Takara) was added in all samples as a control. 24 hr after electroporation, total RNA and cell lysates were prepared. HEK293T cells, cultured in D-MEM-10% FBS, were transiently transfected with the different plasmids by using the jetPei TM Polyplus transfection reagent (Polyplus–TransfectionSA, Società Italiana Chimici, Rome, Italy) according to the manufacturer's protocol. The total amount of DNA for each transfection was kept constant using an empty expression vector.

Quantitative real time RT-PCR

Total RNA was extracted from the cell samples using the RNeasy minikit (#74104, Qiagen). Starting from 1 μg of RNA, first-strand cDNA synthesis was performed with quantiTect reverse transcription kit (#205311, Qiagen). 25 ng of cDNA was amplified with the KAPA SYBR FAST qPCR kit (KK4600, Kapa Biosystem) in accordance to the manufacturer's instructions on an ABI PRISM 7300 Sequence Detection System (Life Technologies). Cycling was performed using the default conditions of the ABI 7300 SDS Software 1.3: 2 min at 95°C, followed by 38 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec a 70°C. The relative expression of *Stra8* was normalized against *Gapdh.* The primers used (n. 14–17) are indicated in **Table 1**.

Reporter assay

1×10^5 HEK293T cells or P19EC cells were seeded in 24 wells and co-transfected with 200 ng of reporter plasmids and with 800 ng of pcDNA3-*Sohlh1* or pcDNA3-S*ohlh*2 and 400 ng of each in the case of co-transfection. Each well received also 10 ng of a pRL-TK Vector (Promega) to normalize for transfection efficiency. After 24 hr, 1 μM RA was added and at 48 hr after transfection, cells were washed 3 times with PBS and scraped in 100 μl of reporter lysis buffer (Promega). Luciferase activity in 20 μl of the cell extracts was quantified using the Dual-Luciferase Reporter assay system (Promega). Each extract was assayed 3 times with a Hidex luminometer (RadTech, Italy). The *Firefly* luciferase activity was divided by *Renilla* luciferase activity and results expressed as the mean ±SEM of 3 experiments.

Spermatogonia isolation

Enriched spermatogonia cell population was obtained from testes of 7 dpp CD1 mice as previously reported by Rossi et al.[48] The cells were cultured for 4 hr in MEM (Life Technologies) with 1 mM lactic acid, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1× nonessential amino acids (all from Sigma-Aldrich), and supplemented with 10% FBS to promote adhesion of somatic cells. Spermatogonia were recovered after centrifugation. Separation of KIT^+ from KIT^- spermatogonia was performed by magnetic-activated cell sorting (MACS) with CD117 conjugated microbeads (Miltenyi Biotech) as previously described.[41]

Chromatin immunoprecipitation (ChIP)

Proteins obtained from at least 10^7 spermatogonia or KIT^+ and KIT^- spermatogonia isolated from testes of 7 dpp mice, were cross-linked to DNA by direct addition to the culture medium of formaldehyde at 1% final concentration for 10 min at 37°C. After sonication, a ChIP assay was performed according to the Abcam protocol. Protein-DNA complexes were immunoprecipitated overnight in the presence of the specific anti-SOHLH1 antibody (Abcam) or rabbit IgGs (Sigma-Aldrich). DNA was purified by phenol/chloroform/isoamylic alcohol extraction and ethanol precipitation, and resuspended in 20 μl of water and used directly for PCR. Primers used were indicated in **Table 2**.

Western blot analysis

Protein extraction was performed in Lysis buffer [(50 mM HEPES, pH 7.9, 15 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, 1% TritonX-100, 0.1% SDS, 0.5 mM dithiothreitol, 10 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mix (Sigma-Aldrich)] for 30 min on ice. Protein extracts (about 30 μg) were subjected to 10% SDS/PAGE electrophoresis and transferred to PVDF Transfer Membrane Hybond Tm (GE Healthcare Europe GmbH). Membranes were saturated with 5% non-fat dry milk in PBS containing 0.1% Tween20 (PBST) for 1

Table 2. Sequences of the primers used in ChIP experiments

	Sequence 5′3′
Stra8 promoter REGION A	Fw CTGGTGCTACCAAAGTCGTG Rev GAGCGCACCATATCAAACT
Stra8 promoter REGION B	Fw TGACAACGGGGGCAGCTTGCAGGAGG Rev CTTGGAGACTCTGCCCCGGGAGG

hr at RT. Incubation with primary antibodies was carried out at 4°C overnight in PBST with 5% BSA. Horseradish peroxidase-conjugated secondary antibody (GE Healthcare Europe GmbH) was used at 1:5000 dilution in PBST for 1 hr at RT. All proteins were detected with an ECL kit (GE Healthcare Europe GmbH) and visualized by chemiluminescence. Primary antibodies were: anti-EGFP mouse (Santa Cruz Technology, 1:1000); anti-Myc mouse (Santa Cruz Technology; 1:1000); anti-SOHLH1 rabbit (Abcam, 1:1000) anti-SOHLH2 guinea pig [gently provided by J.Miyazaki (Osaka University, Japan), 1:500], anti-tubulin rabbit polyclonal (Sigma-Aldrich, 1:1000) and anti-KIT rabbit (see ref. 49).

Statistical analysis

All experiments were replicates at least 3 times. The means tested for homogeneity of variance, and analyzed by ANOVA. The level of significance was set at $P < 0.05$ and $P < 0.01$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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