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Research article

RESEARCH ARTICLE

Environmentally induced epigenetic transgenerational inheritance of altered SRY genomic binding during gonadal sex determination

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Abstract

A critical transcription factor required for mammalian male sex determination is sex determining region on the Y chromosome (SRY). The expression of SRY in precursor Sertoli cells is one of the initial events in testis development. This study was designed to determine the impact of environmentally induced epigenetic transgenerational inheritance on SRY binding during gonadal sex determination in the male. The agricultural fungicide vinclozolin and vehicle control (dimethyl sulfoxide)-exposed gestating females (F0 generation) during gonadal sex determination promoted the transgenerational inheritance of differential DNA methylation in sperm of the F3 generation (great grand-offspring). The fetal gonads in F3 generation males were used to identify potential alterations in SRY binding sites in the developing Sertoli cells. Chromatin immunoprecipitation with an SRY antibody followed by genome-wide promoter tiling array (ChIP-Chip) was used to identify alterations in SRY binding. A total of 81 adjacent oligonucleotide sites and 173 single oligo SRY binding sites were identified to be altered transgenerationally in the Sertoli cell vinclozolin lineage F3 generation males. Observations demonstrate the majority of the previously identified normal SRY binding sites were not altered and the altered SRY binding sites were novel and new additional sites. The chromosomal locations, gene associations and potentially modified cellular pathways were investigated. In summary, environmentally induced epigenetic transgenerational inheritance of germline epimutations appears to alter the cellular differentiation and development of the precursor Sertoli cell SRY binding during gonadal sex determination that may influence the developmental origins of adult onset testis disease observed.

Key words: transgenerational; inheritance; vinclozolin; testis; infertility; developmental origins

Introduction

A number of environmental factors from toxicants to nutrition have been shown to promote the epigenetic transgenerational inheritance of disease and phenotypic variation in a growing number of different species, from plants to humans [1]. One of the first environmental toxicants found to promote the phenomenon was the agricultural fungicide vinclozolin and one of the main transgenerational diseases observed was associated

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with the testis and male infertility [2, 3]. Following the exposure of an F0 generation gestating female, the F3 and F4 generation males had spermatogenic cell apoptosis, reduced sperm number and motility, and increased male infertility [2–4]. Recently, the F3 generation vinclozolin lineage Sertoli cells were found to have an altered transgenerational transcriptome that correlated to the spermatogenic cell defect and testis disease [5].

The critical window for environmental exposure of the F0 generation gestating female was at the end of primordial germ cell (PGC) migration and colonization of the gonad through the onset of gonadal sex determination (embryonic days 8–14 in the rat) [1, 2, 6]. During this period of development, the PGCs have an erasure of the majority of DNA methylation that then at the onset of sex determination is initiated to re-methylate in a male- or female-specific manner to eventually generate the sperm or egg cells [7]. Environmental factors during this period appear to alter this epigenetic programming to promote imprinted-like differential DNA methylation regions (DMR) in the germ cells, termed epimutations [1], that transmit the transgenerational inheritance of germ cell epimutations and disease to subsequent generations [5, 8].

The process of mammalian sex determination was originally outlined by Jost [9–11] with the events of fetal gonadal sex determination followed by phenotypic sex determination. The testis determining factor proposed by Jost [9, 12] was subsequently identified and termed the sex determining region on the Y chromosome (SRY) [13–15]. SRY is a high mobility group (HMG) box transcription factor that binds DNA and can bend DNA [16]. SRY is highly conserved in mammals [14] and other organisms [17]. SRY is generally transiently expressed in Sertoli cells during the initiation of male gonadal sex determination. Subsequent to SRY expression, SOX9 is expressed, another HMG box factor, that also promotes a cascade of transcriptional events required for Sertoli cell differentiation and testis development [18, 19]. The cascade of transcriptional events involved in male gonadal sex determination has previously been studied and reviewed [20–22]. *Sry* is the initial event to promote this cascade of transcriptional events during precursor Sertoli cell differentiation and male gonadal sex determination.

Investigation of the downstream individual gene targets of SRY has identified *Sox9* [18, 19], *Tcf21* [23], *Nt3* [24], and *Cbln4* [25]. Investigation of the downstream targets of *Sox9* initially identified individual genes such as anti-Müllerian hormone [26, 27], fibroblast growth factor 9 [28–30], and others [22]. SOX9 has the capacity to replace SRY binding at many of these sites later in Sertoli cell differentiation [18, 31]. Following the actions of SRY on target transcription factors like SOX9 and TCF21, subsequent cascades of transcriptional events and targets are regulated [23, 32, 33]. Genome-wide analysis of SRY targets in the rat identified 71 binding sites using an SRY chromatin immunoprecipitation followed by a promoter tiling array (SRY ChIP-Chip) [32]. These analyses demonstrate the more global actions of SRY at the onset of Sertoli cell differentiation.

In considering the molecular mechanisms involved in the environmentally induced epigenetic transgenerational inheritance of testis disease, the impacts on Sertoli cell differentiation were investigated. Vinclozolin-induced transgenerational adult onset spermatogenic cell apoptosis and testis abnormalities were found to involve alterations in the adult Sertoli cell transcriptome and epigenome [5]. The F3 generation vinclozolin lineage Sertoli cells had alterations in over 100 differential DMRs and over 400 genes had altered gene expression. Many of the previously identified genes involved in male infertility and testis disease were present in this altered transgenerational transcriptome [5]. Interestingly, the adult Sertoli cell altered gene set had eight genes associated with pyruvate production [5], which is the energy source/metabolite produced for use by the developing spermatogenic cells sequestered within the blood testis barrier [34, 35]. A decrease in pyruvate production would correlate to an increase in spermatogenic cell apoptosis observed [35–38]. Therefore, the epigenetic transgenerational inheritance mechanism appears to involve the germline (sperm) transmission of altered DMR-termed epimutations, such that the embryonic stem cell obtained after fertilization would have an altered epigenome, which would generate an altered epigenome and transcriptome in all cells derived from these stem cells [1, 5]. Later in development, the developing somatic cells will have susceptibility to develop disease due to the altered epigenomes and transcriptomes. This was shown for the Sertoli cells associated with testis disease and the granulosa cells associated with ovary disease in the F3 generation vinclozolin lineage animals compared with the control lineage animals [5, 8].

This study was designed to investigate the potential transgenerational alterations in SRY targets at the onset of Sertoli cell differentiation associated with the initiation of gonadal sex determination. The altered transgenerational Sertoli cell differentiation observed in the adult [2, 3, 5] was speculated to in part be due to induced alterations at the initial stages of Sertoli cell fate determination and differentiation. Previously, we demonstrated an altered testis [39] or PGC [40] transcriptome in vinclozolin F3 generation males, but this study is focused on Sertoli cells. Since SRY is only expressed in Sertoli cells in the fetal testis, potential alterations in SRY binding sites would suggest an altered transcriptional regulation at the onset of Sertoli cell differentiation.

Results

The experimental design involved the intraperitoneal exposure of gestating female rats to vinclozolin or a vehicle control (dimethyl sulfoxide) transiently from embryonic days E8 to E14 [2, 5]. Sister littermates were divided into control and vinclozolin treatment groups and mated to similar males to minimize the genetic variation between the control and vinclozolin lineages. Sufficient females were used so no inbreeding (sibling or cousin) occurred in any generation. The F1 generation was bred within the lineage to generate the F2 generation, and these F2 generation bred to generate the F3 generation as previously described [1, 2, 5]. The only exposure was the F0 generation female. The F3 generation control and vinclozolin lineage embryonic day 13 (E13) embryos were collected and the gonads micro-dissected and then sexed with an SRY polymerase chain reaction (PCR) protocol previously described [41]. The male gonads were pooled from a minimum of three different litters and the pools used to collect DNA. Three different experiments were performed to collect three control and vinclozolin E13 F3 generation testis pools, each with different animals ($n = 25$ gonads/pool). The chromatin DNA (not denatured) from each pool was fragmented and used in an SRY chromatin immunoprecipitation (ChIP) procedure for each pool separately as previously described [32]. The control and vinclozolin SRY ChIP DNA were paired for a competitive hybridization on a genome-wide promoter tiling array (ChIP-Chip) assay. The hybridization data obtained were used to identify the SRY binding sites that were different in the F3 generation vinclozolin versus control lineage in E13 testis as previously described [32].

The number of single oligonucleotide tiling array probe differential SRY binding sites detected in the vinclozolin F3 generation lineage E13 testis was 173, and the number of ≥ 3 adjacent oligo tiling array probe sites was 81, Fig. 1. A minimum

statistical significance of each SRY binding site identification that was different between the control and vinclozolin lineage F3 generation E13 testis was $P < 10^{-4}$, see Methods. The ≥3 adjacent probe sites were selected for further investigation as this is a more stringent selection that avoids the potential false positives and negatives in the single probe sites identified. The complete list of the SRY binding sites is presented in Supplementary Table S1 for ≥3 sites and Supplementary Table S2 for single probe sites. A comparison of the vinclozolin F3 generation E13 testis SRY binding sites with control animal SRY binding sites using the comparative hybridization protocol demonstrated that all the previously identified SRY binding sites [32] were the same in the control with no alterations in the vinclozolin lineage, Fig. 1B. Therefore, the previous sites in the control E13 testis identified [32] were essentially the same in the vinclozolin F3 generation E13 testis, as they had similar competitive hybridization. SRY binding sites identified in this study are altered in the vinclozolin lineage versus the control lineage F3 generation E13 testis precursor Sertoli cells. Therefore, the novel SRY binding sites identified are new additional sites in comparison to the control lineage.

Examples of novel SRY binding site profiles are presented in Fig. 2 for three representative sites. The list of all the 81 ≥3 adjacent probe sites is presented in Supplementary Table S1. The adjacent oligo probe hybridization sites are presented with a $P < 10^{-4}$ statistical difference for each individual probe in the adjacent region. The negative or reduced hybridization previously was identified as non-specific IgG binding sites that can interfere with the detection of positive SRY binding sites [32]. All the SRY binding sites are new positive binding at the regions identified. The chromosome locations of the altered SRY binding sites (≥3 adjacent probes) are presented in Fig. 3. SRY binding sites are present on most autosomes and the X-chromosome. The analysis used a competitive hybridization between the control and vinclozolin F3 generation lineages, and the 71 previously identified SRY binding sites in control animals (≥3 adjacent probe) [32] were not found to be altered in this analysis. The absence of these sites in this study indicates they were not altered in the vinclozolin lineage, Fig. 1B.

The genes associated with the 81 differential SRY binding sites are listed in Table 1 with the gene categories and function indicated. The complete lists of the adjacent and single oligo probe SRY binding sites are presented in Supplementary Tables S1 and S2. The primary gene categories associated with the SRY binding sites are presented in Fig. 4. The main gene categories/functions associated were similar to the gene categories previously shown to be associated with control animal SRY binding sites [32]. A pathway analysis of the SRY binding site-associated genes identified the Immune Influenza A pathway with ≥3 genes associated with the list presented in Table 2. The single oligo SRY-altered binding site pathways involved are presented in Table 3. The vinclozolin induced epigenetic transgenerational inheritance of testis disease appears to have involved an alteration in SRY binding sites during the initial differentiation of Sertoli cells and onset of testis determination.

Discussion

Environmentally induced epigenetic transgenerational inheritance of disease and phenotypic variation involves the germline transmission of epigenetic information, in the absence of direct exposure [1, 42–55]. Since the mature germ cell (sperm or egg) transmits the altered epigenome to the embryonic stem cells, all cells derived from the embryonic stem cells will have an altered epigenome and transcriptome [1, 5, 8]. Each cell type will have a unique cascade of epigenetic and genetic events that leads to cell specific differentiation involving a unique epigenome and transcriptome [56]. Therefore, epigenetic transgenerational inheritance impacts all somatic cells which will alter the organism's phenotype and in those tissues sensitive to the alteration potential disease susceptibility [1]. Because of the dependence of this phenomenon on the germ cell, the hypothesis was tested that a cell at the onset of its differentiation and cell fate determination will have a shift in its normal transcriptional events leading to the cell's differentiation.

The Sertoli cell is one of the key somatic cells in the testis that supports the development and function of the developing germ cells undergoing spermatogenesis to develop into sperm in the epididymis [57–59]. In the adult, Sertoli cells from the seminiferous tubule provide the complex structural support for the developing spermatogenic cells from the spermatogonia stem cell state through the release of spermatozoa. During this process, the Sertoli cells provide the microenvironment and nutrient support required [58, 59], such that abnormalities in Sertoli cell function can lead to testis abnormalities and male infertility [60, 61]. In addition to the role of Sertoli cells in the adult male, Sertoli cells are also essential for the initial stages of germ cell development and gonadal sex determination [62–64]. When the PGCs migrate down the genital ridge and initially colonize the indifferent gonad, the PGC and somatic cells are in an undifferentiated state prior to the onset of gonadal sex determination. The somatic cells associated with the PGC in the gonad at the onset of sex determination initiate their cell fate determination to promote the PGC development into a male or female germline lineage associated with the initial testis or ovary development, termed gonadal sex determination and initially discussed by Jost [9–11]. Therefore, as observed in the adult, the precursor Sertoli cell in the initiation of testis development and conversion of the PGC to a male germ line lineage is critical for germ cell development and differentiation.

The initial event in cell fate determination and cellular differentiation of Sertoli cells involves the testis determining factor [13–15] identified as SRY. The SRY is an HMG-box protein that binds and can bend DNA to regulate gene expression. One of the initial downstream targets of SRY is the *Sox9* gene that can initiate a cascade of molecular events to promote the differentiation of Sertoli cells [18, 19]. The onset of Sertoli cell differentiation by SRY marks the induction of gonadal sex determination of the testis and male germ cell differentiation. A number of downstream targets for SRY have been identified, with *Sox9* induction being thought to be one of the primary regulatory events [18–22]. Previous studies have shown SRY can target the genes encoding an array of proteins such as SOX9 [18, 19] and TCF21 [23] that in turn can promote a subsequent cascade of transcriptional events [33]. For example, using a ChIP-Chip analysis for the rat, SOX9 was found to target 109 genes [32] and TCF21 targeted 121 genes [33]. Therefore, if an environmental factor could alter SRY genome targets, the abnormal epigenetic and developmental cascade would be expected to influence Sertoli cell differentiation. This would then persist to the adult Sertoli cell state and potentially associate with testis abnormalities and disease [5].

This study used the agricultural fungicide vinclozolin to promote the epigenetic transgenerational inheritance of testis disease into the F3 generation following a transient exposure of a pregnant F0 generation female during gonadal sex determination [2]. Previously, the adult F3 generation males have been shown to have spermatogenic cell apoptosis and male infertility [2–4]. The

A

SRY ChIP-Chip	Number Single Sites	Number Adjacent ≥3 Sites	P Value
F3 Generation E13 Testis	173	81	$1x10^{-4}$

B

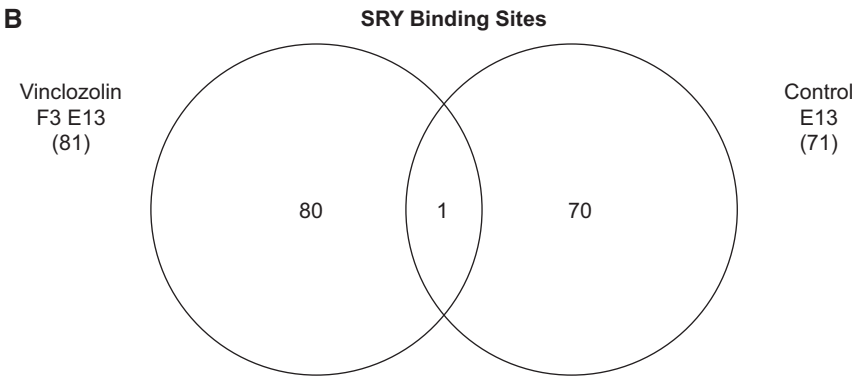

Figure 1. SRY binding sites. (A) Vinclozolin lineage F3 generation E13 testis for single oligo probe detection and ≥3 adjacent oligo probe detection. (B) Venn diagram overlap of ≥3 adjacent site SRY binding sites identified in the vinclozolin F3 generation E13 testis versus the normal control E13 testis previously identified [32]

embryonic day 13 (E13) testis from control and vinclozolin lineage F3 generation male rats was used for this study. The E13 stage in the rat correlates with the early stage of Sertoli cell differentiation and testis development when SRY has induced the expression of SOX9 and other target genes [32, 40]. The chromatin obtained from the E13 testis was used in a ChIP-Chip analysis with the SRY antibody to identify target binding sites for SRY. This was analyzed on a genome-wide promoter tiling array with a competitive hybridization of the control versus vinclozolin F3 generation E13 testis DNA using two different fluorescent labels. Observations indicated 81 altered SRY targets using a high stringency selection of three adjacent oligo probes with each having a statistically significant change in hybridization. With a less stringent single oligo probe analysis, 173 SRY binding sites were detected. To reduce the issue of false positives and negatives, the 81 SRY targets found to be different between the control versus vinclozolin lineage samples were used for further analysis. The 71 SRY binding target genes previously identified in control normal E13 testis [32] were not different between the control versus vinclozolin lineage samples. Therefore, the majority of the normal Sertoli cell developmental pathways and SRY targets were not affected, but 81 new and novel SRY targets were identified. The environmentally induced epigenetic transgenerational inheritance of the altered cellular transcriptomes and epigenomes in Sertoli cells did not alter the normal developmental molecular events but added additional molecular targets. Epigenetics has the ability to directly alter transcription factor binding and gene expression.

The gene associations of these altered SRY binding targets demonstrated a variety of affected gene functional categories. Pathway analysis identified the influenza A pathway affected with greater than four genes being the most significant pathways influenced by the altered SRY targets. These potential effects on the early Sertoli cell differentiation may indirectly influence the developing germ cells. Therefore, the gene targets associated with the altered SRY binding may have a significant impact on gonadal sex determination and eventually the differentiated state of adult Sertoli cells and spermatogenic cells.

A previous study demonstrated the adult Sertoli cell in the vinclozolin lineage F3 generation male had an altered epigenome and transcriptome compared with the control lineage males [5]. A gene pathway that was affected involved pyruvate production that is required for the developing germ cells within the blood tests barrier [34–38]. The reduction in pyruvate production may be directly correlated with the dramatic increase in spermatogenic cell apoptosis observed in the adult testis of vinclozolin lineage males [2, 3]. This study suggests the alterations observed in the adult Sertoli cell of vinclozolin lineage originated in part at the initiation of Sertoli cell fate determination during gonadal sex determination. Therefore, the developmental origins of the adult onset testis disease were in part identified. Although the disease does not develop until later in life, the developmental origins occurred at the initial cell fate determination.

In regards to the initial hypothesis tested, observations support the role of the germline in transmitting an altered epigenome to subsequently affect the developing embryonic stem cell epigenome that then impacts all derived somatic cell epigenomes and transcriptomes. The impact on all cells and tissues has the ability to alter a large number of disease states. Indeed, the vinclozolin lineage transgenerational animals have a large number of disease conditions observed [1–3] and phenotypic variation in areas such as behavior [65, 66]. The current observations indicate a direct impact on sex determination, key transcriptional events of SRY and its function, and somatic cell function involving Sertoli cell—germ cell interactions. Since the environment induced these alterations at a critical stage of development, environmentally induced epigenetic transgenerational inheritance of diseases and phenotypic variation is anticipated to have a critical role in biology.

Methods

Tissue Preparation

Harlan Sprague-Dawley rats were used for the study. All the rats were kept in a temperature controlled environment. Estrous cycles of female rats were monitored by cellular morphology from vaginal smears. Rats in early estrus were paired

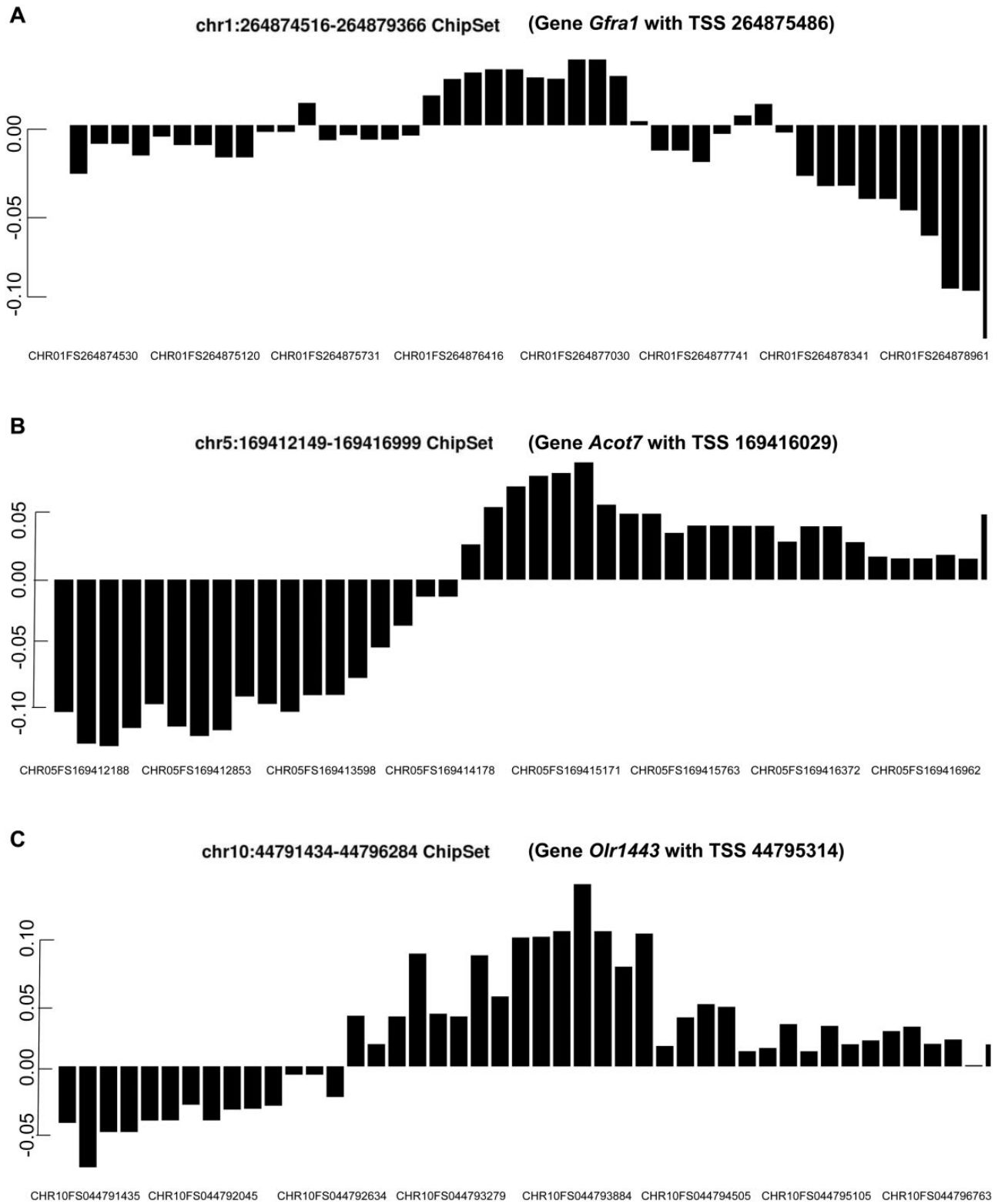

Figure 2. Representative SRY binding site profiles on (A) Chromosome 1, (B) Chromosome 5, and (C) Chromosome 10. Each probe hybridization is presented (bar) with the positive peak indicating the SRY binding site. The location of the SRY binding site is indicated with the positive peak. The gene and transcriptional start site (TSS) is listed for each

with males overnight and mating confirmed by sperm positive smears, denoting day 0 of pregnancy. The breeding of control and vinclozolin lineage F0, F1, and F2 generations were as previously described [2–6]. The F0 generation females were exposed to vinclozolin or dimethyl sulfoxide through IP injection daily from E8 to E14 [2–6]. The F2 generation pregnant rats were euthanized at gestational embryonic day 13 (E13) of pregnancy, and F3 generation embryonic gonads were collected for

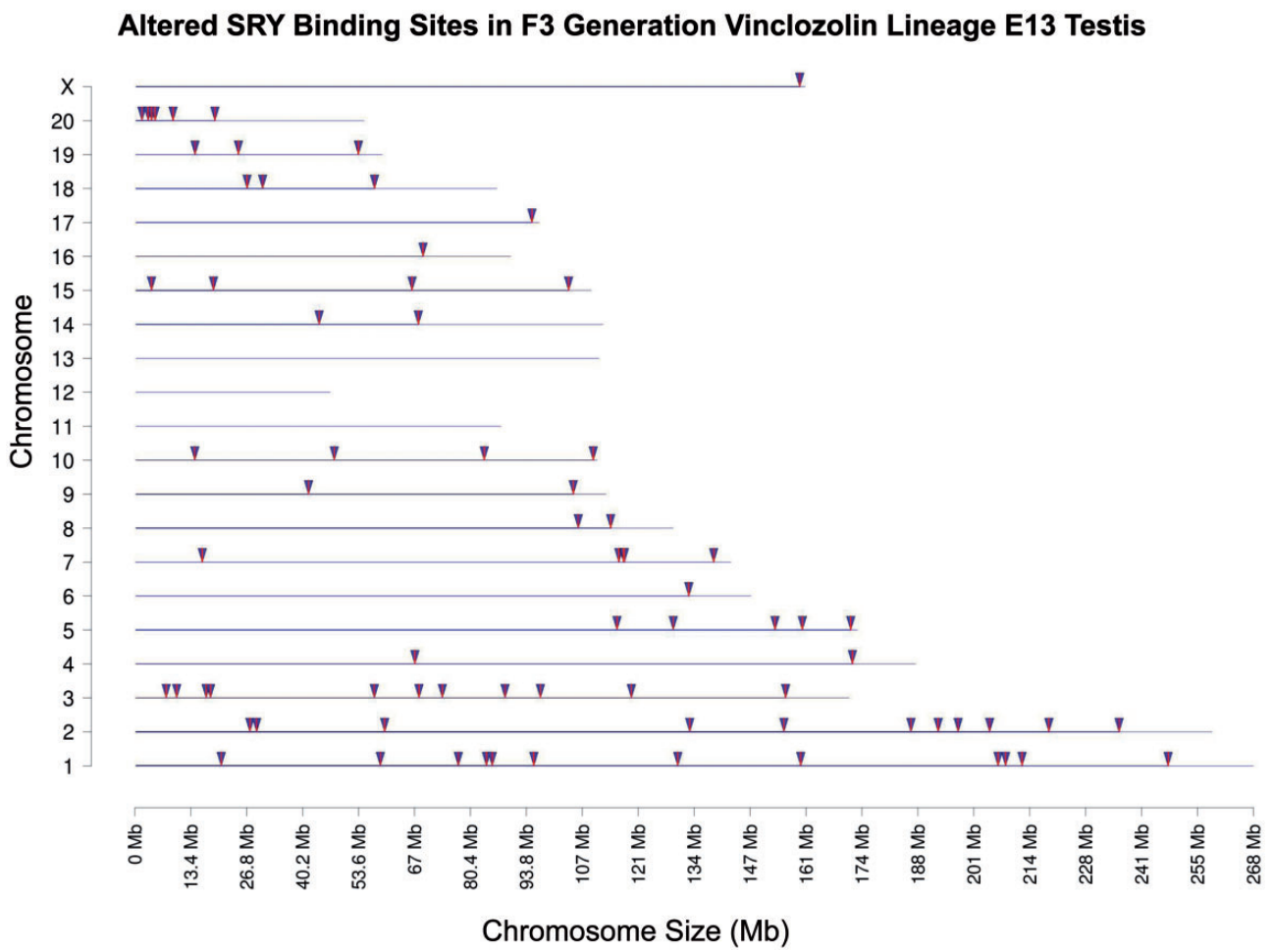

Figure 3. Chromosomal location of the SRY binding sites. The chromosome number (y-axis) versus chromosome size (mb) (x-axis) is shown with the red arrow heads indicating SRY binding sites in the altered SRY binding sites F3 generation vinclozolin lineage E13 testis

chromatin immunoprecipitation. A somite count of 12–18 tail was also used to help stage the fetus for this developmental period as previously discussed [32, 33]. Sex was determined by PCR using primers specific for *Sry* on genomic DNA isolated from embryo tails as previously described [41]. All procedures were approved by the Washington State University Animal Care and Use Committee (IACUC approval no. 02568-034).

In Vivo ChIP Assay

A modified ChIP (cChIP) assay was adopted from O'Neill et al. [67] and performed according to Bhandari et al. [23]. The conditions for the native-ChIP (not including cross linking) were optimized for immunoprecipitating with SRY antibodies. The native-ChIP was used to identify high affinity binding sites and reduce low affinity sites. To run a replicate of the ChIP assay, at least 25 male gonads from thirty 13 dpc (12–18 tail somite stage) rat embryos were used per array. All three ChIP experiments were with different biological samples. *Drosophila* SL2 cells (American Type Culture Collection (ATCC) Catalog no. CRL-1963) were used as a carrier. Densely grown cells ($\sim 5 \times 10^7$ cells) were pelleted and washed three times in ice-cold phosphate-buffered salve, 5 mM sodium butyrate, and resuspended in 0.5 ml NB buffer [15 mM Tris-HCL, pH 7.4, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.5 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonylfluoride (PMSF)]. Testis samples were mixed with SL2 cells and homogenized to make single cell suspension. Nuclei were pelleted, resuspended in 10 ml NB buffer, 5% (vol/vol) sucrose, pelleted, and resuspended again in 1.5 ml digestion buffer (50 mM Tris-HCl pH 7.4, 0.32 M sucrose, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF). Following micrococcal nuclease digestion (NEB, USA) for 5 minutes at 28°C, the digested samples were gently spun (800 x g) for 15 minutes and supernatant set aside on ice. The pellet was resuspended in 250 µl digestion buffer and again centrifuged gently at 800 x g for 15 minutes at 4 degrees. Both the supernatants were pooled, and a fraction (50 µl) out of it was kept aside to use as input. The remaining supernatant was incubated with either non-immune IgG or anti-SRY (Santa Cruz, CA) antibody at 4°C overnight. The specificity of these antibodies on western blots have been previously described and validated [23].

After incubation with 100 µl of pre-swollen protein A-Sepharose beads (SL2 DNA blocked) for 2 h at 4°C, the bead-bound immunoprecipitates were centrifuged gently and washed five times with wash buffer (50 mM TrisHCl pH 7.5, 10 mM EDTA, 5 mM Na butyrate, and 50–150 mM NaCl). The protein–DNA complexes were incubated at room temperature with elution buffer (1% SDS in TE) and centrifuged at 11 500 x g for 10 minutes. Elution was repeated two times, and eluted DNA was pooled. Co-immunoprecipitated DNA was purified by phenol/chloroform extraction and ethanol precipitation. Final

Table 1. SRY binding associated genes and gene function

Gene name	NCBI_gene ID	Gene function	Chr	Cluster ID	Probes (no.)	Length bp
Il18bp	84388	Signaling	chr1	chr1:159475918–159478113	3	2196
Tigd3	309174	Development	chr1	chr1:208529885–208532090	3	2206
Rab3il1	171452	Signaling	chr1	chr1:212473968–212476572	6	2605
Hpse2	368128	Metabolism	chr1	chr1:247461058–247463393	4	2336
Vom1r19	494298	Receptor	chr1	chr1:58783093–58785503	3	2411
Psg16	308394	Development	chr1	chr1:77466423–77469105	8	2683
Lce1l	686125	Development	chr2	chr2:185871514–185873696	3	2183
Slc25a24	310791	Transport	chr2	chr2:204716693–204718991	3	2299
RGD1309170	362047	Unknown	chr2	chr2:235717341–235719802	5	2462
Tor1b	311854	Metabolism	chr3	chr3:10018125–10020621	3	2497
RGD1561852	499893	Unknown	chr3	chr3:118912719–118914915	3	2197
Slc43a1	311168	Transport	chr3	chr3:68044469–68046640	3	2172
Olr694	295896	Receptor	chr3	chr3:73637127–73639417	4	2291
Cat	24248	Metabolism	chr3	chr3:88649222–88651529	4	2308
Olr766	405356	Receptor	chr3	chr3:97122942–97125269	3	2328
Eif3s6ip	300069	Translation	chr7	chr7:117061657–117064644	10	2988
Uqcrc1	301011	Metabolism	chr8	chr8:113951092–113953366	3	2275
Actg1	287876	Cytoskeleton	chr10	chr10:109774391–109776911	5	2521
Epn2	60443	Transport	chr10	chr10:47741753–47743932	3	2180
Ppp1r9b	84686	Cytoskeleton	chr10	chr10:83669966–83672713	5	2748
Uchl1	29545	Metabolism	chr14	chr14:44123206–44125426	3	2221
Dnajc3	63880	Metabolism	chr15	chr15:103881239–103883433	3	2195
Pxk	306203	Signaling	chr15	chr15:18817572–18819962	5	2391
Pcdh17	306055	Cell adhesion	chr15	chr15:66387053–66389494	5	2442
Chrna6	81721	Receptor	chr16	chr16:69007355–69009564	3	2210
Pkd2l2	291683	Transport	chr18	chr18:26871615–26873827	3	2213
Ppargc1b	291567	Transcription	chr18	chr18:57382172–57384447	3	2276
Cdk10	361434	Cell cycle	chr19	chr19:53540561–53542901	4	2341
RT1-DMb	294273	Immunity	chr20	chr20:4838459–4841177	6	2719
Zfp275	293849	Transcription	chrX	chrX:159237289–159239865	7	2577

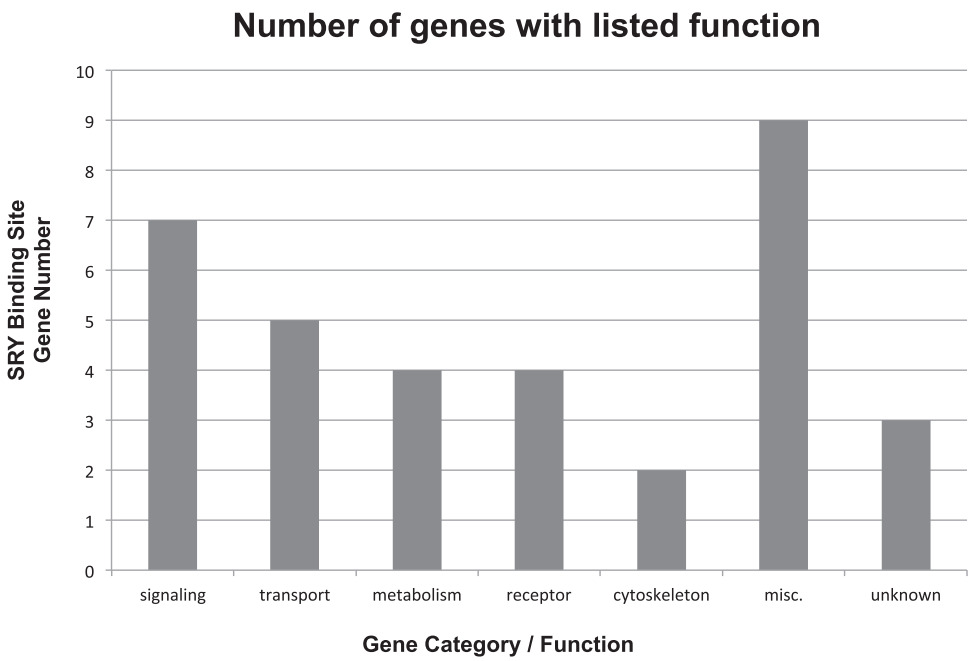

Figure 4. Gene categories and function for SRY binding sites. The number of genes associated with various gene categories is presented

concentration of immunoprecipitated DNA varied from 200 to 500 ng per assay. Three different experiments and ChIP assays were performed. Exactly 30 ng of immunoprecipitated DNA from each assay was amplified by whole-genome amplification kit developed by Sigma (Sigma no. WGA2 50 RXN). At least five separate whole-genome amplifications were performed, and DNA was pooled. Pooled whole-genome amplified DNA was purified by using Promega's Wizard SV40 PCR cleanup kit

Table 2. Cellular pathways associated with altered SRY binding sites: the 81 ≥3 adjacent oligo SRY binding sites

	Influenza A pathway (3 altered)
Actg1	Actin, gamma 1
RT1-DMb	RT1 class II, locus DMb
Dnajc3	DnaJ (Hsp40) homolog, subfamily C, member 3

Table 3: Cellular pathways associated with altered SRY binding sites: the 173 single oligo SRY binding sites

	Metabolic pathways (7 altered)
Pnp	Purine nucleoside phosphorylase (EC:2.4.2.1)
Ppcs	Phosphopantothenoylcysteine synthetase (EC:6.3.2.5)
Uqcrc1	Ubiquinol-cytochrome c reductase core protein I (EC:1.10.2.2)
Cers2	Ceramide synthase 2
Hpse2	Heparanase 2
Fut4	Fucosyltransferase 4 (alpha (1,3), myeloid-specific) (EC:2.4.1.152)
Mthfd2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2,
	Olfactory transduction pathways (7 altered)
Olr1443	Olfactory receptor 1443
Olr694	Olfactory receptor 694
Olr848	Olfactory receptor 848
Olr1285	Olfactory receptor 1285
Olr766	Olfactory receptor 766
Olr51	Olfactory receptor 51
Olr1654	Olfactory receptor 1654
	MicroRNAs in cancer pathways (4 altered)
Ddit4	DNA-damage-inducible transcript 4
Notch1	Notch 1
Brca1	Breast cancer 1, early onset
Cdca5	Cell division cycle associated 5

(Promega). Purified DNA was checked on the gel and sent to Nimblegen for ChIP-Chip hybridization (Nimblegen, Iceland). A three plex array (3x720 RefSeq Promoter Array) was used for hybridization comparisons. The ChIP-Chip advanced technology used is more accurate and statistically stringent than the more classic PCR technology, such that we do not feel PCR of selected targets helps validate the data.

Bioinformatics Analysis of ChIP-Chip Data

The ChIP-Chip hybridization used a Roche Nimblegen's Rat ChIP-Chip 3x720K RefSeq Promoter Array. The enrichment for each probe on the array was calculated as the log ratio of the intensities of hybridization for SRY ChIP DNA (Cy3) to control DNA from IgG control (Cy5). Array contained on average 4000 bp of promoter for each of 15 287 promoters in the rat genome corresponding to 15 600 RefSeq transcripts (~3880 bp upstream and 970 bp downstream from transcription start site). The analysis of ChIP-Chip data was performed as previously described [68]. For each hybridization experiment, raw data from both the Cy3 can Cy5 channels were imported into R [44] and data checked for quality and converted to MA values ($M = \text{Cy5-Cy3}$; $A = (\text{Cy5} + \text{Cy3})/2$). The R codes that were used for analysis and annotation are available in the following website: http//www.skinner.wsu.edu. All the tiling array Chip data were deposited in the NCBI GEO site (GEO # GSE72469).

Within each array, probes were separated into groups by GC content, and each group was separately normalized using the LOESS normalization procedure [69]. This allowed for groups with optimal GC content, which exhibited a reduced quality issue, to receive a normalization curve specific to that group. After each array was normalized within array, the arrays were then normalized across arrays using the A-quantile normalization procedure [70]. Following normalization, the probe's normalized *M* values (and then A) were replaced with the median value of all probe *M* values (and then A) within a sliding window of 1000 bp [71–73], due to the size of DNA fragments used. Following normalization, each probe's *M* value represents the median intensity difference between Cy5 and Cy3 of a 1000 bp window. Significance was assigned to probe differences between experimental (SRY) and IgG control by calculating the median value of the intensity differences when compared with a normal distribution scaled to the experimental mean and standard deviation of *M*. Regions of interest were then determined by combining consecutive probes with significance *P* values $< 10^{-3}$. Significance was assigned to probe differences between experimental and control by calculating the median value of the increasing differences when compared with a normal distribution scaled to the experimental mean and standard deviation of the mean. A *Z* score and *P* value were computed from that distribution with the use of R code analysis. The statistical package was similar to the edgeR bioconductor package and used empirical Bayes methods [74]. The statistically significant peaks of hybridization were identified and *P* value associated with each peak presented. Each peak of interest was then annotated for the gene. Every promoter exceeding the intensity threshold was considered positive for SRY binding. The final list of SRY targets includes the promoter-proximal regions that made the threshold in an average of the three replicates. Hybridization signals for all the candidate promoters that were within the cutoff line ($P \leq 1 \times 10^{-4}$) were plotted (average of the three replicates). The genes that were not in the list but seemed to be masked by IgG negative signals were designated as questionable positives.

Gene Network and Pathway Analysis

Gene network analysis identified groups functionally interconnected genes whose expression is linked to cellular processes. In this study, gene networks for SRY downstream binding target genes were constructed separately using previously published criteria for developmental network analysis [75]. Global literature analysis of various gene lists was performed with Pathway Studio software using BiblioSphere Pathway Edition (Genomatix Software GmbH, Munchen, Federal Republic of Germany), which performs direct gene interaction (connection) analysis and relationship with cellular processes (indirect interactions).

The cellular signaling pathway analysis of direct downstream target genes was performed according to the protocol previously described [75]. The downstream binding targets of SRY and their associations with pathways were analyzed for KEGG (Kyoto Encyclopedia for Genes and Genome, Kyoto University, Japan) pathway enrichment using Pathway-Express, a web-based tool freely available as part of the Onto-Tools (http://vortex.cs.wayne.edu). A program based on literature analysis Pathway Studio (Ariadne, Genomics Inc., Rockville, MD) was used to evaluate cellular processes connected to binding targets associated genes. The analysis of statistical overrepresentation of genes within a pathway used a Fisher's Exact

test using 2 × 2 contingency table. The pathway analysis is distinct from the gene network analysis described above.

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Supplementary data

Supplementary data is available at *EnvEpig* online.

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