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Transgenerational Epigenetic Imprinting of the Male Germline by Endocrine Disruptor Exposure during Gonadal Sex Determination

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Embryonic exposure to the endocrine disruptor vinclozolin at the time of gonadal sex determination was previously found to promote transgenerational disease states. The actions of vinclozolin appear to be due to epigenetic alterations in the male germline that are transmitted to subsequent generations. Analysis of the transgenerational epigenetic effects on the male germline (*i.e.* sperm) identified 25 candidate DNA sequences with altered methylation patterns in the vinclozolin generation sperm. These sequences were identified and mapped to specific genes and noncoding DNA regions. Bisulfite sequencing was used to confirm the altered methylation pattern of 15 of the candidate DNA sequences. Alterations in the epigenetic pattern (*i.e.* methylation) of these genes/DNA sequences were found in the F2 and F3 generation germline. Therefore, the reprogramming of the male germline involves the induction of new imprinted-like genes/DNA sequences that acquire an apparent permanent DNA methylation pattern that is passed at least through the paternal allele. The expression pattern of several of the genes during embryonic development were found to be altered in the vinclozolin F_1 and F2 generation testis. A number of the imprinted-like genes/DNA sequences identified are associated with epigenetic linked diseases. In summary, an endocrine disruptor exposure during embryonic gonadal sex determination was found to promote an alteration in the epigenetic (*i.e.* induction of imprinted like genes/DNA sequences) programming of the male germline, and this is associated with the development of transgenerational disease states. (*Endocrinology* 147: 5524–5541, 2006)

PREVIOUSLY WE demonstrated that embryonic exposure to an endocrine disruptor at the time of gonadal sex determination caused a transgenerational effect on male fertility and testis function (1). Interestingly, more than 90% of all males from all subsequent generations analyzed (F1–F4) had a reduced spermatogenic capacity. The initial transgenerational disease state observed has recently been expanded to the development of a wide variety of other disease states as the animals age including tumor development, prostate disease, and kidney disease (2). The high frequency of disease prevalence (20–50%) in affected animals cannot be attributed to DNA sequence mutations (3, 4) that generally occur at a frequency less than 0.01%. Therefore, an epigenetic mechanism involving altered DNA methylation of the germline was proposed (1). The ability of an environmental factor to epigenetically reprogram the male germline to induce transgenerational disease states is investigated in the current study through the identification of imprinted-like genes/DNA sequences in sperm. The term imprinted-like gene/DNA sequence is defined here as a DNA sequence that acquires an altered DNA methylation pattern that is transmitted transgenerationally (F1-F3) through at least the male germline (*i.e.* paternal allele).

During embryonic development the primordial germ cells migrate down the genital ridge and colonize the indifferent bipotential gonad before sex determination (5, 6). As the primordial germ cells migrate down the genital ridge, their genomic DNA is demethylated such that the germ cells genome before sex determination is not methylated (7). At the onset of sex determination, the germ cell DNA is remethylated in a sex-specific manner (8). Somatic cells in the developing gonads are required for normal germ cell development and DNA methylation (9–12). Although the majority of the genome is demethylated and remethylated after fertilization and early embryonic development, a small subset of genes are imprinted and transmit a unique methylation pattern to subsequent generations through the male or female germline (13). Modification of the DNA methylation pattern of previously identified imprinted genes has been shown to induce disease states (14). Therefore, alterations in the epigenetic programing (*i.e.* DNA methylation) of the germline when the cell is establishing its methylation pattern at the onset of sex determination could induce an epigenetic transgenerational disease state.

Endocrine disruptors are a class of environmental and therapeutic compounds that bind to hormone receptors, alter hormone signaling, and influence (*i.e.* disrupt) the endocrine system (15). These compounds include a wide variety of commonly used substances from pesticides to plastics. Previous studies have demonstrated that exposure to a number of different endocrine disruptors during embryonic or early

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Abbreviations: E, Embryonic day; Fadd, Fas associated via death domain; MSRE, methylation-sensitive restriction enzymes; Mup4, major urinary protein 4; NCAM1, neural cell adhesion molecule 1; SD, Sprague-Dawley; Six, sine oculis homeobox homolog; snRP1c, small nuclear ribonucleoprotein subunit; Waspip, Wiskott-Aldrich syndrome protein interacting protein.

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postnatal development can cause adult onset disease states including reproductive defects (16) and tumor development (17). For example, the therapeutic agent diethylstilbestrol after embryonic exposure causes abnormal female and male reproductive tract development (18, 19), including some F2 generation defects being identified (20). Recently two endocrine disruptors, the fungicide vinclozolin (antiandrogenic) and pesticide methoxychlor (estrogenic, antiestrogenic, and antiandrogenic), have been shown to promote male germ cell defects for multiple generations (F1–F4) after exposure of an F0 gestating mother during the time of gonadal sex determination (1). The ability of an environmental compound (endocrine disruptor) to induce a transgenerational disease state in the progeny of an exposed gestating mother, impacts the potential hazards of these compounds and suggests novel mechanisms in disease etiology.

The phenomena of the fetal basis of adult onset disease is centered on the concept that embryos are more sensitive to environmental factors and alterations in embryonic development can cause the development of a variety of disease states in the adult (21). Examples of factors that can promote adult-onset disease include environmental toxicants (22, 23), abnormal maternal physiology (22), and nutritional support of the embryo (24). Recently observations suggested some of these adult-onset disease states may be transgenerational (1). The only mechanism known to transmit a transgenerational heritable disease state requires the involvement of the germline. The potential that an epigenetic alteration of the germline could be a factor in adult-onset disease has not been previously appreciated. The hypothesis tested in the current study is that embryonic exposure to an environmental factor (endocrine disruptor) at the time of gonadal sex determination induces a permanent alteration in the epigenetic reprogramming (DNA methylation) of the germline that promotes transgenerational disease states. The current study identifies a set of new imprinted-like genes/DNA sequences that are involved in the epigenetic reprogramming of the male germline and appear to be associated with the development of transgenerational epigenetic disease. Although the cause and/or effect of the imprinted-like genes/DNA sequences identified remains to be determined, the novel observation is provided that an environmental compound can induce a transgenerational change in the epigenome of the germline.

Materials and Methods

Sperm collections

Rats (Fischer and Sprague-Dawley strains) were treated using vinclozolin (100 mg/kg·d) from embryonic d (E) 8–14 of gestation as previously described (1, 25). In brief, four lines (individual F0 treated females) were generated for control and four lines were generated for vinclozolin experimental groups. F1 vinclozolin generation males were bred to F1 vinclozolin generation females to generate the F2 vinclozolin generations and F2 vinclozolin generation animals bred to generate the F3 vinclozolin generation animals. Rats for the control groups were bred in the same way for all the generations. No sibling breeding occurred to avoid any inbreeding artifacts. Male rats from control and vinclozolin generation animals were collected at postnatal d 60–180 for analyses. Animals were euthanized and cauda epididymal sperm was collected for further experiments. Briefly, the cauda epididymis was dissected and placed in prewarmed F12 media containing 1% BSA for 30 min at 37 C as previously described (25). Sperm were collected from the media for DNA isolation. The inbred strain of Fischer rat was used for the initial methylation screen to identify the candidate methylation sites due to the lack of DNA polymorphisms. To confirm the methylation changes observed, the outbred strain of Sprague-Dawley (SD) rats was used for the bisulfite analysis and to investigate altered methylation between F1 and F3 generations and for the microarray gene expression data.

DNA methylation assay

The methylation state of the DNA isolated from inbred strain of Fischer control and vinclozolin F3 generation epididymal sperm was determined using a combination of methylation-sensitive restriction enzymes (MSRE) and PCR procedures previously described (1). Briefly, genomic DNA was isolated from sperm samples using the DNeasy tissue kit (QIAGEN, Valencia, CA). Two micrograms each of control and vinclozolin generation sperm DNA were separately digested with *Rsa*I and either methylation-sensitive enzyme *Hpa*II or insensitive *Msp*I enzymes, followed by PCR with specific primer sets as previously described (supplemental Table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). PCR products were electrophoretically separated on polyacrylamide gels and visualized by ethidium bromide staining. The PCR products (*i.e.* bands) that were reproducibly absent or present between control and vinclozolin generation sperm DNA were excised from stained gels, reamplified with the same PCR primer set, and then cloned and sequenced. The DNA sequence and chromosomal location was determined using the National Center for Biotechnology Information rat genomic database BLAST system.

Bisulfite sequencing

Bisulfite sequencing was used to characterize the DNA methylation patterns and changes. Genomic DNA was isolated from SD control and vinclozolin F2 and F3 generation epididymal sperm using the DNeasy tissue kit. Genomic DNA (10 μg) was digested with *Rsa*I and then treated with bisulfite as previously described (1). After purification, the bisulfite-converted DNA was used as a template for PCR. The bisulfite primers for each candidate were designed using the MethPrimer (www.urogene.org/methprimer) system (26) and listed in supplemental Table S1. The sequence-specific primers were generated to amplify the CpG region of interest to characterize the methylation pattern. PCR conditions for amplification were as follows: 30 cycles followed by another 30 cycles with nested primers (30 sec at 95 C, 45 sec at 50 C, and 50 sec at 72 C) and then 5 min at 72 C with modified 1× buffer [10 times: 166 mM $(NH_4)_2.SO_4$; 670 mM Tris (pH 8.8); 67 mM $MgCl_2$; 100 mM β-mercaptoethanol]. PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced using Big-Dye Terminator (Applied Biosystems, Foster City, CA). Approximately 50 different clones from each PCR product were sequenced to characterize the methylation state of the CpG sites identified. A mixture of a minimum of three different F2 or F3 generation control and vinclozolin animals were used to generate approximately 50 clones per candidate analyzed. Statistical differences between methylation states were determined with a Fisher's exact test.

Genomic DNA and methylation-sensitive restriction enzyme PCR

Genomic DNA was isolated from the different SD control and vinclozolin F1–F3 generation epididymal sperm samples. A total of 1 μg of genomic DNA was digested using methylation-sensitive *Hpa*II or *Aci*I enzymes (37 C, 6–12 h). The digested DNA was precipitated and dissolved in 30 μl dH_2O. Then 3 μl aliquots were analyzed by PCR in 30-μl reactions with specific primers (supplemental Table S1), subjected to 25 cycles, and the PCR products electrophoretically separated on 1.5% agarose. The difference between the control and vinclozolin F1–F3 generation sperm samples was determined for selected genes found to have high optimal digestion capacity.

Microarray analysis

RNA was collected from E13, E14, and E16 testis from control and E16 vinclozolin F1 and F2 generation SD animals as previously described (27, 28). RNA was hybridized to the rat 230A gene chip (Affymetrix, Santa

Clara, CA). The Genomics Core in the Center for Reproductive Biology at Washington State University performed the analysis as previously described (27, 28). Briefly, RNA from the cells was reverse transcribed into cDNA and then was transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the Affymetrix rat 230A gene chips. Each gene set is composed of 16 pairs of 24-mer oligonucleotides, with one sense strand-specific for the gene and one antisense strand with single-point mutations for use as comparative negative control. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray was scanned on a gene array scanner (Hewlett-Packard Co., Palo Alto, CA). Two microarray chips from two different RNA samples were analyzed for each E13, E14, and E16 control and E16 vinclozolin F1 and F2 generation testis. The microarray data set can be observed online (www.skinner.wsu.edu).

Bioinformatics

The majority of the data analysis was performed using the Affymetrix Microarray Suite software. Most of this study used the comparison analysis software offered and analysis parameters previously described (28). Two repeats for each control and vinclozolin F1 and F2 generation E16 testis was performed and allowed four comparisons in the experiment. Only genes that displayed a consistent expression over all four comparisons and had a relative hybridization intensity of at least 50 were included in the analysis. Basic expression analysis was determined by accessing the Affymetrix database through the Microarray Suite software. A pathway analysis was performed by the Pathway Assist Gene Spring software (Silicon Genetics, Foster City, CA).

Results

Gestating rats were transiently exposed to the antiandrogenic endocrine disruptor vinclozolin during embryonic gonadal sex determination (E8–E14) as previously described (1). Subsequent F1, F2, and F3 generation progeny from control and vinclozolin-treated F0 mothers were produced. The F0 mothers used for the control (vehicle treatment, n = 4) and vinclozolin treatment (n = 4) were littermate sisters such that the genetic background of the control and vinclozolin generations were similar. The control and vinclozolin generation animals were maintained in the same conditions (*i.e.* room, rack, feed) but kept in different cages. The control and vinclozolin (F1–F3) generation males were euthanized as adults (*i.e.* 90–180 d of age) and their epididymal sperm collected and DNA isolated. These sperm DNA samples were used to investigate the potential epigenetic changes (*i.e.* DNA methylation) between the control and vinclozolin generation male germline.

The F3 generation samples were used to identify alterations in DNA methylation between control and vinclozolin generation sperm. This analysis assures that any changes observed are due to a transgenerational epigenetic transmission and not due to toxicology that could be present in the F1 generation. The analysis of methylation used a MSRE procedure that involved the use of 10 different sets of PCR primers to consensus CpG methylation sites (MSRE-PCR) (29) as described in *Materials and Methods*. Sperm DNA from control and vinclozolin F3 generation animals (*i.e.* Fischer rats) from three different preparations containing a minimum of three different animals were analyzed. A representative gel for one set of PCR primers is shown in Fig. 1 with the three control and vinclozolin samples shown. Differences in the MSRE *Hpa*II (H) digest demonstrated the presence of PCR products (bands) in the vinclozolin samples, but not control samples (Fig. 1). If differences in the PCR products (bands) occurred in a minimum of two of the three replicate animals, they were selected for further analyses. This analysis generated 34 candidates with potential altered DNA methylation between control and vinclozolin sperm samples and were numbered 1–34 (Table 1). The representative differences for candidates 19–22 are shown in Fig. 1 and the other gels and candidates in supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Interestingly, all candidates selected were present in the vinlcozolin samples but not in the control samples. There were no candidates that were present in the control sample and absent in the vinclozolin samples. The PCR product (*i.e.* band) for all the candidates were excised from the gels, subcloned, and then sequenced. The characterization of the candidates is shown in Table 1. The presence of more than one candidate number together (*e.g.* 2, 3, 24) indicates similar sequences being present in the different candidates. The gene(s) nearest the candidate sequences are listed with GenBank accession numbers. The physical map (*i.e.* location) of the candidate DNA methylation site is shown as being in the promoter, exon, intron, or distal noncoding region of DNA (Table 1). The sequence was considered in the promoter of a gene if it was within 1000 bp upstream of the transcriptional start site of the gene of interest.

The chromosomal location of all the candidate DNA methylation sequences is shown in Fig. 2. The candidate altered methylation sites are present on various autosomes with no major hot spot regions and none present on the sex chromosomes. The rat genome is not complete so several candidate sequences could not be localized and appear to be on uncharacterized chromosomal regions (Fig. 2). Therefore, the MSRE-PCR screen identified 25 individual candidate DNA sequences with potential altered methylation sites that are different between control and vinclozolin generation F3 sperm DNA samples (Table 1). As discussed below, some of these candidates fall within the promoters, exons, or introns

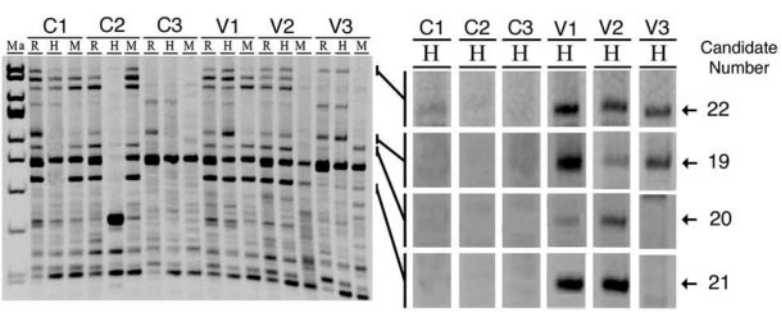

FIG. 1. DNA methylation analysis from control (C) and vinclozolin (V) F3 caudal epididymal sperm. Representative gel image of the PCR-based methylation sensitive *Hpa*II+*Rsa*I (H lanes), insensitive *Msp*I+*Rsa*I (M lanes), and control *Rsa*I (R lanes) as digests. Bands that appeared to be differentially methylated between matched control and vinclozolin samples are enlarged for H bands and indicated by *arrows* for the specific candidates 19, 20, 21, 22. The size DNA 100-bp ladder is in the *left lane* (Ma). These bands were isolated and sequenced, and the gels for all other candidates are presented in supplemental Fig. S1.

TABLE 1. Identification of 25 candidates isolated from MSRE-PCR screen

Candidate no.	Location	Identification (rat accession no.)
1	Exon/intron	*NCAM1* (NM_031521)/similar to phosphoglycerate mutase B chain LOC503205 (XM_573155)
2, 3, 24	Noncoding region	Leucine-rich protein 157 (*Lrpprc*) (NM_001008519)/protein phosphatase 1B (*Ppm1b*) (NM_033096)
4, 19	Intron	Calcium channel, voltage-dependent, L type, α1E subunit (*Cacna1e*) (NM_019294)
5, 6, 9	Exon/intron	Similar to FLJ22405 protein (XM_232266)
7	Noncoding region	Similar to ankyrin repeat domain protein 28 (*Ankrd28*) (LOC306264) (XW_224620.3)/polypeptide *N*-acetylgalatosaminyl transferase (*Galntl2*-predicted) (NW_047469)
8	Noncoding region	Liprin-α1 (*Ppfia1*) (XM_341856) /*Fadd* (TNFRSF6) (NW_047563)
10	Unknown	No significant similarity found
11	Noncoding region	60S ribosomal protein L7a (LOC502773) (NW_047693)/RIKEN cDNA 1200009022 (LOC362364) (NM_001034010))
12	Promoter	Similar to importin 7 (LOC501910) (NC_005113)
13	Intron	Nuclear factor I/X (*Nfix*) (AB012234)
14, 15	Noncoding region	Leucine rich repeat neuronal 6A (*Lrrn6a*) (XM_236268)
16	Promoter/exon/intron	Optineurin (*Optn*) (NM_145081)
17, 18	Promoter	Nicastrin (*Ncstn*) (NM_174864)/coatomer protein complex subunit-α (*Copa*) (XM_222899)
20	Unknown	No significant similarity found
21, 32	Exon/intron	Similar to phosphoglycerate mutase B chain (LOC503205) (XM_573155)/*NCAM1* (NM_031521)
22	Intron	Similar to golgi autoantigen golgin subtype a4 (*tGolgin-1*) (LOC501069) (XM_236718)
23	Noncoding region	Similar to ribosomal protein (LOC503150) (XP_578671.1) *snRNP1c* (LOC503151) (XP_578672)
25	Noncoding region	*Mup4* (NM_198784)/similar to major urinary protein precursor (MUP 2u-globulin) (LOC502951) (XM_578456)
26	Promoter	Acetyl-coenzyme A carboxylase α (*Acaca*) (NM_022193)
27, 28	Intron	Zinc finger protein 212 (*Zpf212*) (XM_231749)
29	Noncoding region	Runt related transcription factor 1 (*Runx1*) (NM_017325)/similar to cell wall protein *Awa1p* (LOC501772) (XP_577171)
30	Unknown	No significant similarity found
31	Noncoding region	*Waspip* (NM_057191)/hypothetical protein (LOC499811) (XP_580019)
33	Noncoding region	*Six3* (NM_023990)/Six2 predicted (XP_345631)
34	Promoter	*RAB12* (ras GTP-binding protein *Rab12*), member RAS oncogene family (XM_343639)

of specific genes, whereas others are noncoding regions distal to a gene and the nearest gene(s) are listed (Table 1).

Verification that these candidates (Table 1) have specific alterations in DNA methylation requires bisulfite sequencing analysis. Bisulfite converts cytosine residues to thymine residues unless the CpG is methylated at the 5′ position on the cytosine, which inhibits the conversion. Specific bisulfite primers were used to amplify the bisulfite converted DNA followed by sequencing of individual clones to assess CpG sites for their methylation state. The bisulfite primers used for each candidate are listed in supplemental Table S1, and the analysis of each of the candidates is described below. Each candidate analysis involved a minimum of three different individual control and vinclozolin F2 and F3 animals with approximately 50 different subcloned DNA samples sequenced. The bisulfite analysis for each individual candidate containing a statistically significant change in methylation is shown in Fig. 3. The chromosomal location of the methylation site and nearest genes are presented, along with the DNA sequence of the candidate site with potential methylated CpG sites *underlined*, and the *asterisks* denote specific CpG with statistically different methylation states between control and vinclozolin generation samples. The methylation state for all bisulfite sequence analyses for all the different clones is represented as *closed circle* for methylated and *open circle* for unmethylated CpG sites.

Candidate characterization

Candidate 1 mapped to 8q23 and is in the exon/intron region of the recently annotated hypothetical gene LOC503205,

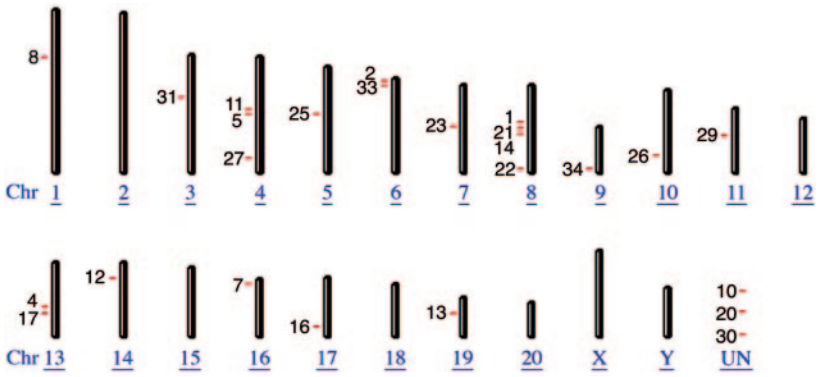

FIG. 2. The chromosome location and physical mapping of each candidate (*numbers*) are indicated for each chromosome (Chr), with those unknown (UN) sequences not mapped indicated.

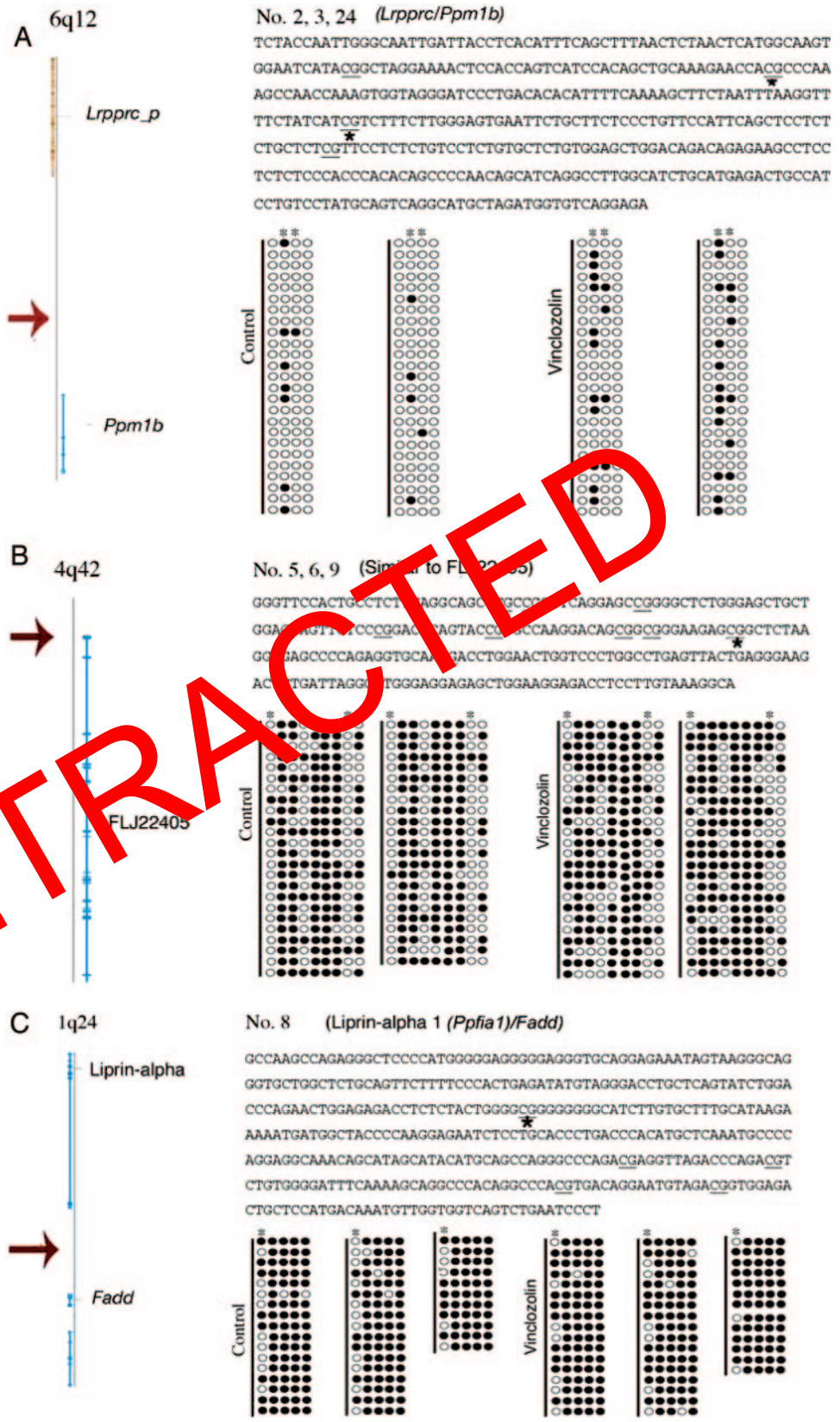

FIG. 3. Methylation status of the candidates with bisulfite sequencing. Each analysis provides the DNA sequence of interest, the physical chromosome map with alignment (*arrow*), and bisulfite sequence results. The potential methylated cytosine residues are marked with an *underline*, and the *asterisks* (*) denote the altered methylated CpG sites. The bisulfite sequencing is presented as a series of *circles* representing the CpG sites *underlined* with an *open circle* (○) denoting nonmethylation and *closed circle* (●) methylation. Each series of *circles* represents individual cloned DNA sequences analyzed. The CpG with statistically significant ($P < 0.05$) altered methylation in the vinclozolin generation sperm DNA is marked with an *asterisk* (*).

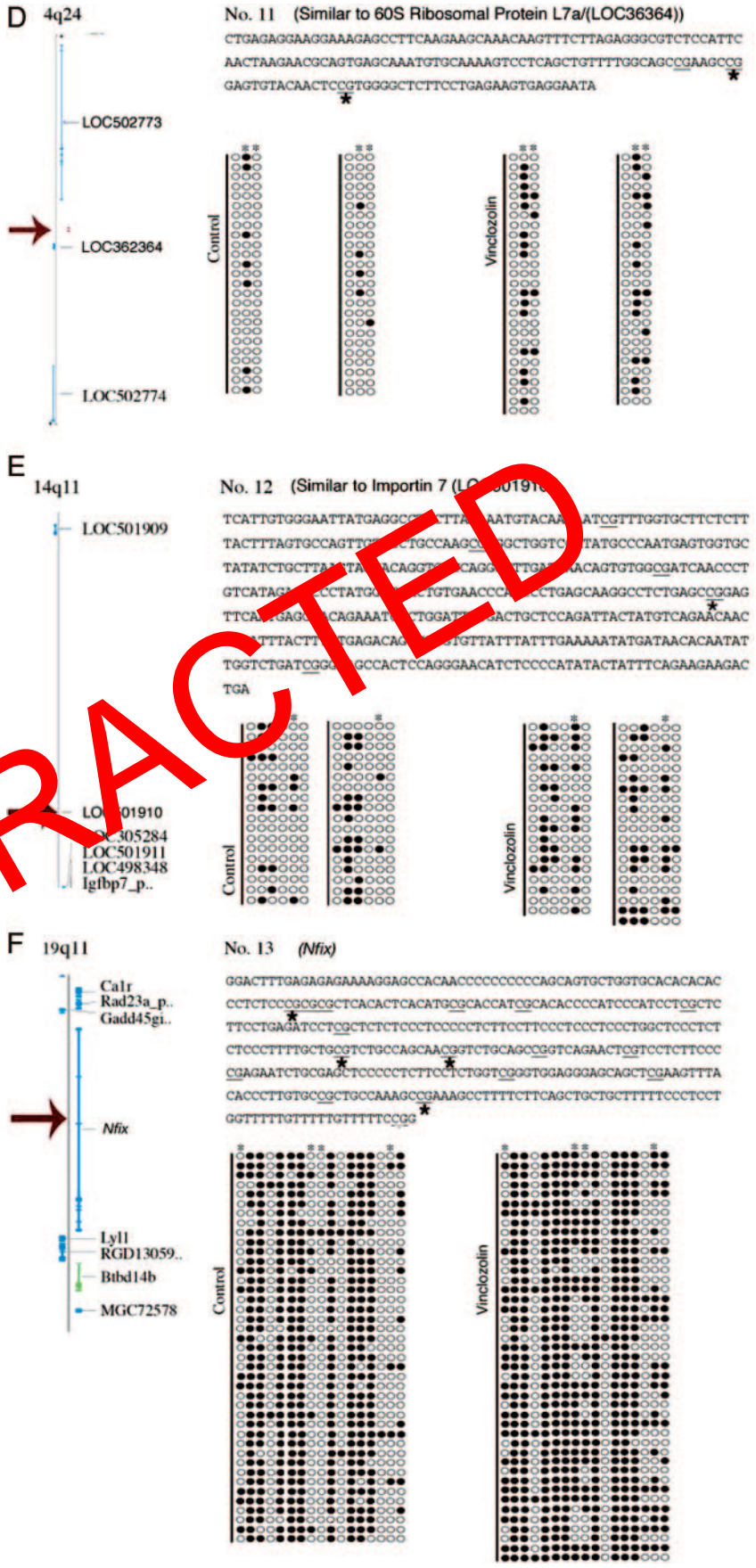

FIG. 3. *Continued*

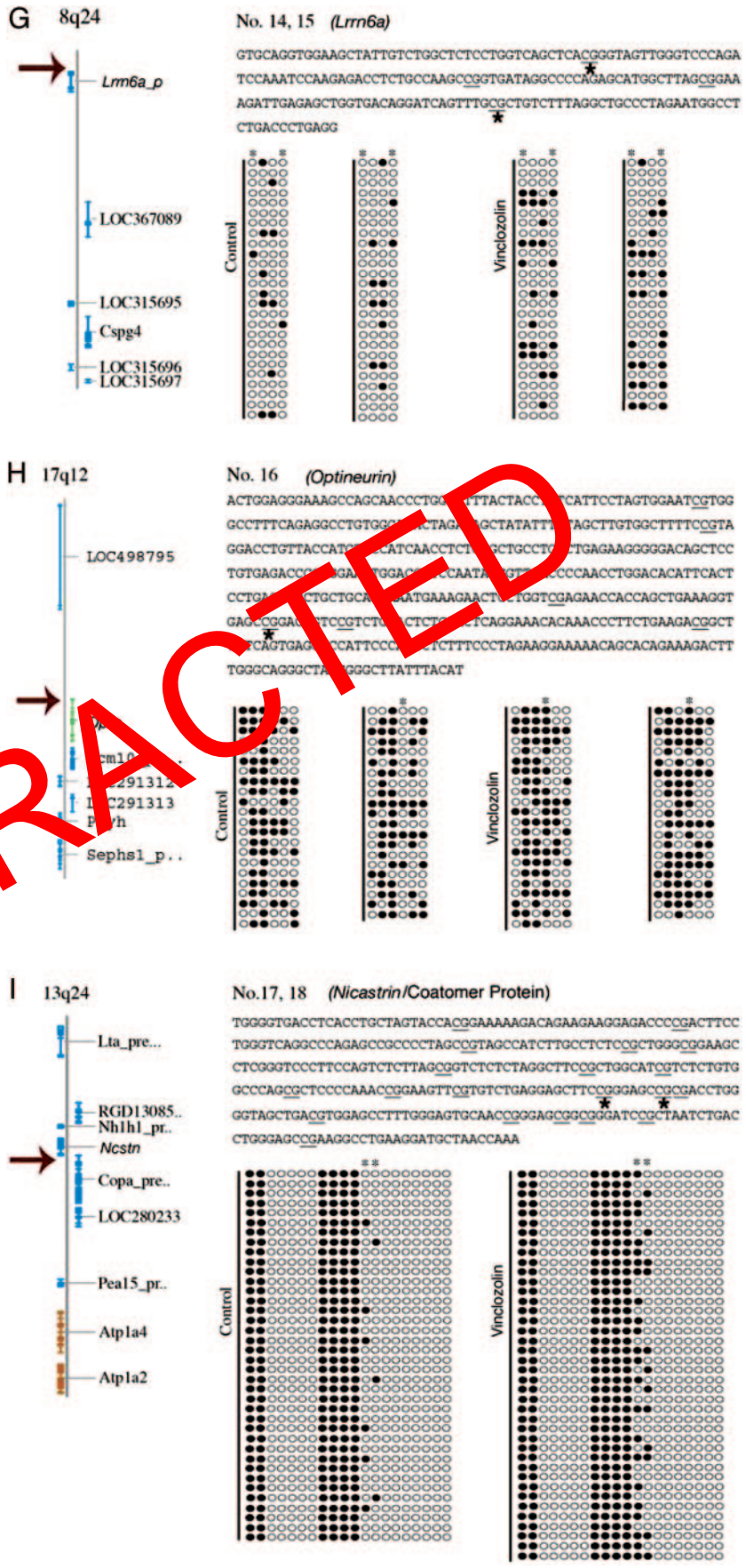

FIG. 3. *Continued*

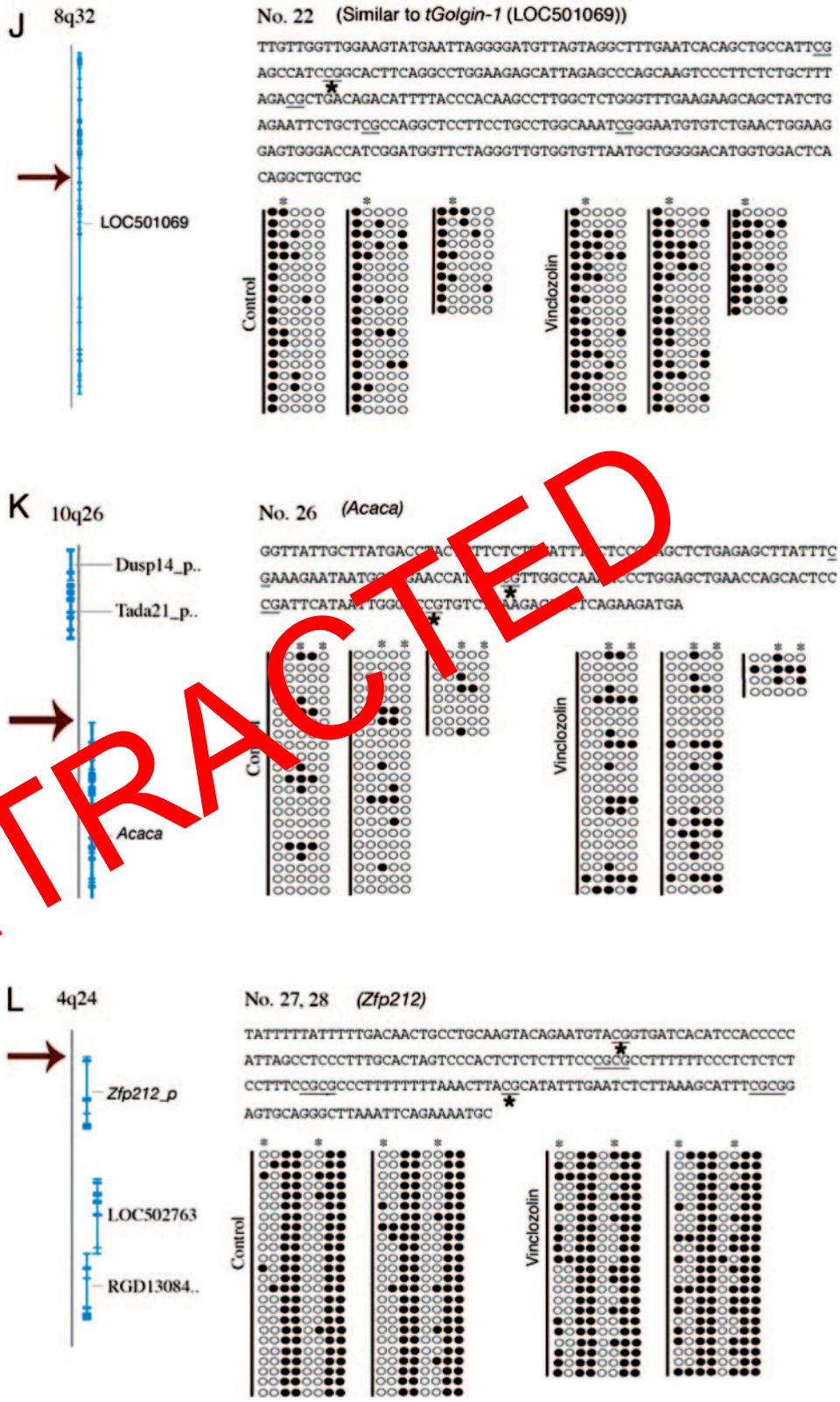

FIG. 3. *Continued*

similar to phosphoglycerate mutase chain B, with the nearest known gene being neural cell adhesion molecule 1 (NCAM1) (supplemental Fig. S3A). Although four potential CpG sites exist, the high GC content of this DNA sequence prevents the design of specific bisulfite primers such that bisulfite methylation analysis was not possible (supplemental Fig. S3A).

Candidates 2, 3, and 24 mapped to 6q12 and are in a

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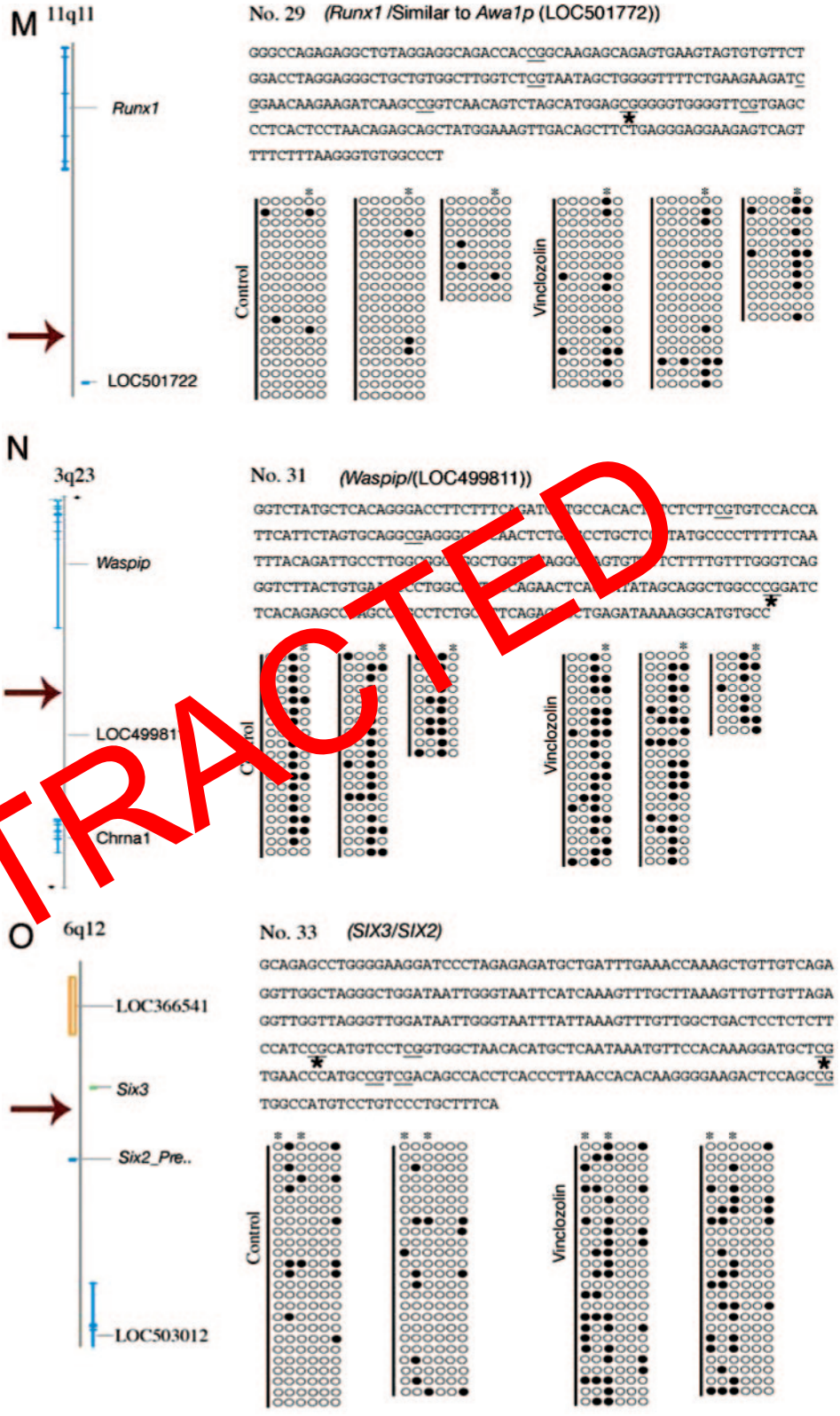

FIG. 3. *Continued*

noncoding distal region with the nearest genes being leucine rich protein 157 (Lrpprc) and protein phosphatase 1B (Ppm1b) (Fig. 3A). The bisulfite methylation analysis revealed four potential CpG sites and two are hypermethylated (*i.e.* statistically different) to 44 and 20% (Fig. 4 and supplemental Fig. S2).

Candidates 4 and 19 mapped to 13q21 and are in the intron of calcium channel, voltage-dependent L type α1E subunit

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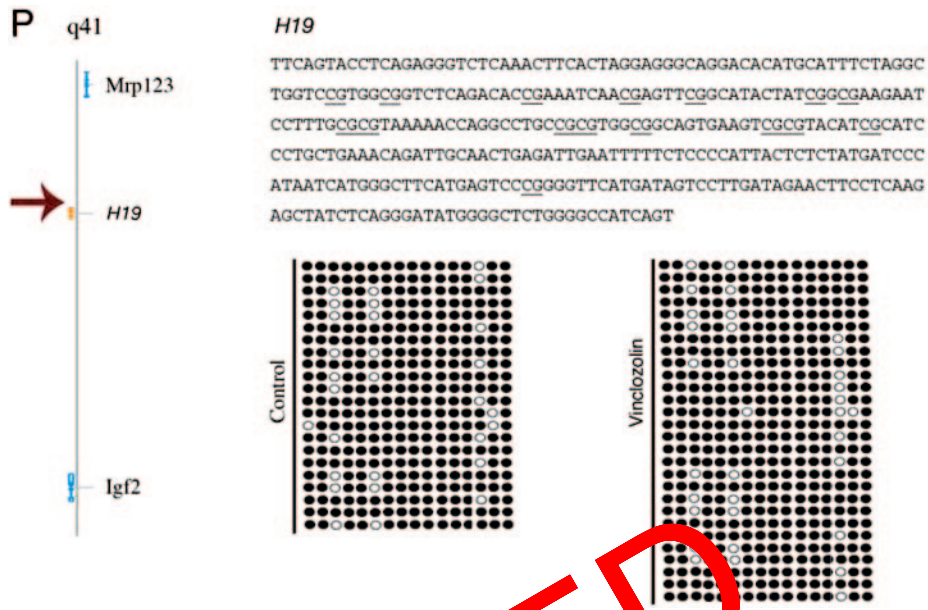

FIG. 3. *Continued*

(Cacnale) (supplemental Fig. S3B). The bisulfite methylation analysis revealed six potential CpG sites that did not have any statistical differences in methylation (supplemental Fig. S3).

Candidates 5, 6, and 9 mapped to 4q42 and are in the exon/intron of an uncharacterized gene FLJ22405 protein (Fig. 3B). The bisulfite methylation analysis revealed nine potential CpG sites and two were hypermethylated (*i.e.* statistically different) to 50 and 35% (Fig. 4 and supplemental Fig. S2).

Candidate 7 mapped to 16q1[illegible] and is in a noncoding region distal to ankyrin repeat protein 28 (Ankrd28) and polypeptide *N*-acetylgalactosaminyl transferase (Galnt12) (supplemental Fig. S3C) and did not have any detectable differences in methylation.

Candidate 8 mapped to 1q42 and is in a noncoding region distal to liprin-α1 and Fas (TNFRSF6) associated via death domain (Fadd) (Fig. 3C). The bisulfite methylation analysis revealed five potential CpG sites and one was hypermethylated (*i.e.* statistically different) to 65% (Fig. 4 and supplemental Fig. S2).

Candidate 10 did not map to a specific chromosome and is uncharacterized (Fig. 2), so methylation with bisulfite analysis was not possible (supplemental Fig. S3D).

Candidate 11 mapped to 4q24 and is in the noncoding region distal to 60S ribosomal protein L7a and uncharacterized RIKEN cDNA 1200009022 (Fig. 3D). The bisulfite methylation analysis revealed three potential CpG sites and two were hypermethyylated (*i.e.* statistically different) to 49 and 21% (Fig. 4 and supplemental Fig. S2).

Candidate 12 mapped to 14q11 and is in the promoter to the hypothetical gene similar to importin 7, LOC501910 (Fig. 3E). The bisulfite methylation analysis revealed six potential CpG sites and one was hypermethylated to 51% (Fig. 4 and supplemental Fig. S2).

Candidate 13 mapped to 19q11 and is in the intron of Nfix protein (Fig. 3F). The bisulfite methylation analysis revealed 17 potential CpG sites and four were hypermethylated to 50–60% (Fig. 4 and supplemental Fig. S2).

Candidates 14 and 15 mapped to 8q24 and are in the

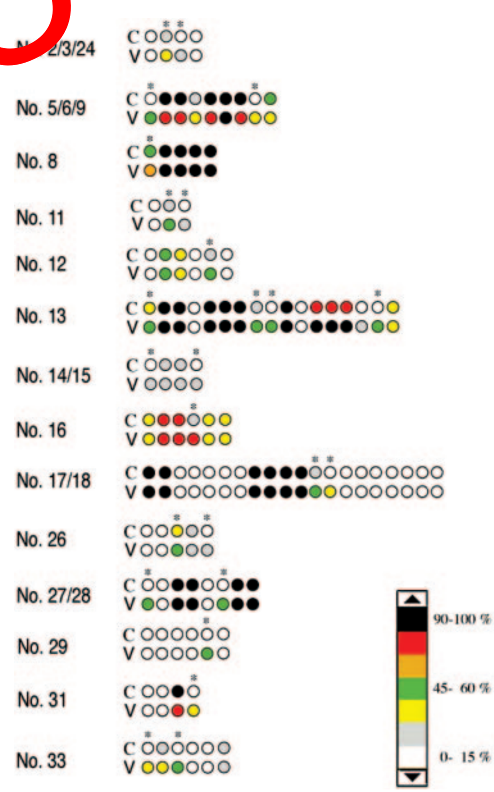

FIG. 4. Summary of altered methylation changes determined by bisulfite sequencing for each confirmed candidate. The percent methylation is presented as *color* in the *circle* for control (C) or vinclozolin (V) F2/F3 generation sperm DNA. The *asterisks* (*) denote statistically significant ($P < 0.05$) different methylation changes, and each *circle* represents the specific CpG sites in the DNA sequence. Specific data are presented in supplemental Fig. S3.

noncoding region distal to leucine rich repeat neuronal 6A (Lrrn6a) (Fig. 3G). The bisulfite methylation analysis revealed four potential CpG sites and two were hypermethylated to 27% (Fig. 4 and supplemental Fig. S2).

Candidate 16 mapped to 17q12 and is in the promoter/exon/intron of optineurin, Fig. 3H). The bisulfite methylation analysis revealed 6 potential CpG sites and one was hypermethylated to 79% (Fig. 4 and supplemental Fig. S2).

Candidates 17 and 18 mapped to 13q24 and are in the bidirectional promoter to nicastrin and coatomer protein complex subunit-α (Fig. 3I). The bisulfite methylation analysis revealed 20 potential CpG sites and two were hypermethylated to 55 and 30% (Fig. 4 and supplemental Fig. S2).

Candidate 20 did not map to a chromosome and is uncharacterized (Fig. 2), so methylation with bisulfite analysis was not possible (supplemental Fig. S3E).

Candidates 21 and 32 mapped to 8q23 and are in the exon/intron region of the recently annotated hypothetical gene similar to phosphoglycerate mutase B chain, LOC503205 (supplemental Fig. S3F), with no differences in methylation after bisulfite analysis. The nearest known gene is NCAM1. This potential methylation site is distinct from candidate 1 but in the same hypothetical gene.

Candidate 22 mapped to 8q32 and is in the intron of tgolgin-1 (Fig. 3J). The bisulfite methylation analysis revealed five potential CpG sites and one was hypermethylated to 67% (Fig. 4 and supplemental Fig. S2).

Candidate 23 mapped to 7q22 and is in the noncoding region distal to a hypothetical gene similar to ribosomal protein, LOC503150, and U1 small nuclear ribonucleoprotein subunit (snRP1c) (supplemental Fig. S3G), with no statistical difference in methylation after bisulfite sequencing.

Candidate 25 mapped to 5q24 and is in the noncoding region distal to major urinary protein 4 (Mup4) and hypothetical gene major urinary precursor, LOC502951 (supplemental Fig. S3H), with no statistical difference in methylation detected with bisulfite analysis. However, the candidate 25 sequence is a repetitive sequence present in 12 different locations in chromosome 5q24. Potential methylation changes in the other locations remains to be investigated.

Candidate 26 mapped to 10q26 and is in the promoter of acetyl-co enzyme A carboxylase-α (Acaca) (Fig. 3K). The bisulfite methylation analysis revealed five potential CpG sites and two were hypermethylated to 50 and 28% (Fig. 4 and supplemental Fig. S2).

Candidates 27 and 28 mapped to 4q24 and are in the intron of zinc finger protein 212 (Fig. 3L). The bisulfite methylation analysis revealed eight potential CpG sites and two are hypermethylated to 45 and 48% (Fig. 4 and supplemental Fig. S2).

Candidate 29 mapped to 11q11 and is in the noncoding region distal to Runx1 and similar to cell wall protein Awa1p (LOC501772) (Fig. 3M). The bisulfite methylation analysis revealed six potential CpG sites and 1 was hypermethylated to 48% (Fig. 4 and supplemental Fig. S2).

Candidate 30 did not map to a chromosome and is uncharacterized (Fig. 2), so methylation with bisulfite analysis was not possible (supplemental Fig. S3I).

Candidate 31 mapped to 3q23 and is in the noncoding region distal to Wiskott-Aldrich syndrome protein interacting protein (Waspip) and hypothetical protein, LOC499811 (Fig. 3N). The bisulfite methylation analysis revealed four potential CpG sites and one was hypermethylated to 42% (Fig. 4 and supplemental Fig. S2).

Candidate 33 mapped to 6q12 and is in the noncoding region distal to sine oculis homeobox homolog (Six) 3 and predicted Six2 (Fig. 3O). The bisulfite methylation analysis revealed six potential CpG sites and two were hypermethylated to 31 and 57% (Fig. 4 and supplemental Fig. S2).

Candidate 34 mapped to 9q37 and is in the promoter of the GTP-binding protein RAB12 and member of the Ras oncogene family (supplemental Figure S3J). Due to the high GC content of this DNA sequence, bisulfite PCR primers could not be designed to analyze the entire sequence such that methylation analysis was not possible except for a partial region, which showed no change in methylation (supplemental Fig. S3J).

Therefore, of the 25 candidate DNA sequences with altered methylation in vinclozolin generation sperm, 15 were confirmed to have specific hypermethylation as summarized in Fig. 4 and supplemental Fig. S2. Six of the candidates were characterized, but no alterations in DNA methylation were detected (supplemental Fig. S3). Whether methylation occurred in the regions proximal to those analyzed remains to be investigated. These candidates are speculated to have altered DNA methylation due to the procedure used in their isolation but remain to be confirmed with bisulfite analysis. Two candidates, no. 1 (NCAM1/similar to phosphoglycerate mutase) and 34 (RAB12) could not be fully analyzed due to high GC content and will require a different technology to confirm the methylation state of the region of interest.

As a control a known imprinted gene H19 (30) was analyzed to determine whether methylation changes were induced. No change in H19 methylation was detected between control and vinclozolin F2/F3 sperm DNA samples (Fig. 3P).

The control and vinclozolin generation sperm DNA used in the initial MSRE-PCR and bisulfite analysis was from F2 and F3 generation males. Therefore, the epigenetic changes observed in the male germline appear transgenerational and permanently programmed into the sperm DNA. MSRE analysis for specific candidate DNA sequences was developed (Fig. 5). The MSRE analysis involved gene-specific PCR primers to the candidates to obtain a PCR product with the vinclozolin generation sperm DNA *vs.* control. Analysis of sperm DNA from F1, F2, and F3 control and vinclozolin generation animals demonstrated candidates 1, 14/15, 17/18, 27/28, and 33 all show similar PCR results for F1-F3 sperm (Fig. 5). PCR products were observed in all the vinclozolin generation sperm DNA samples but not in the controls. Therefore, the methylation changes identified are transgenerational and appear imprinted-like in the male germline. Due to the variability in methylation shown in Figs. 3 and 4, many of the candidates and DNA sequences analyzed could not be used to develop a specific MSRE analysis (data not shown). One of the candidates (no. 33) did not have a consensus *Hpa*II digestion site but did reproducibly digest the DNA (Fig. 5), and another candidate (no. 27/28) used an alternate methylation sensitive restriction enzyme *Aci*I. Combined observations demonstrate the epigenetic changes

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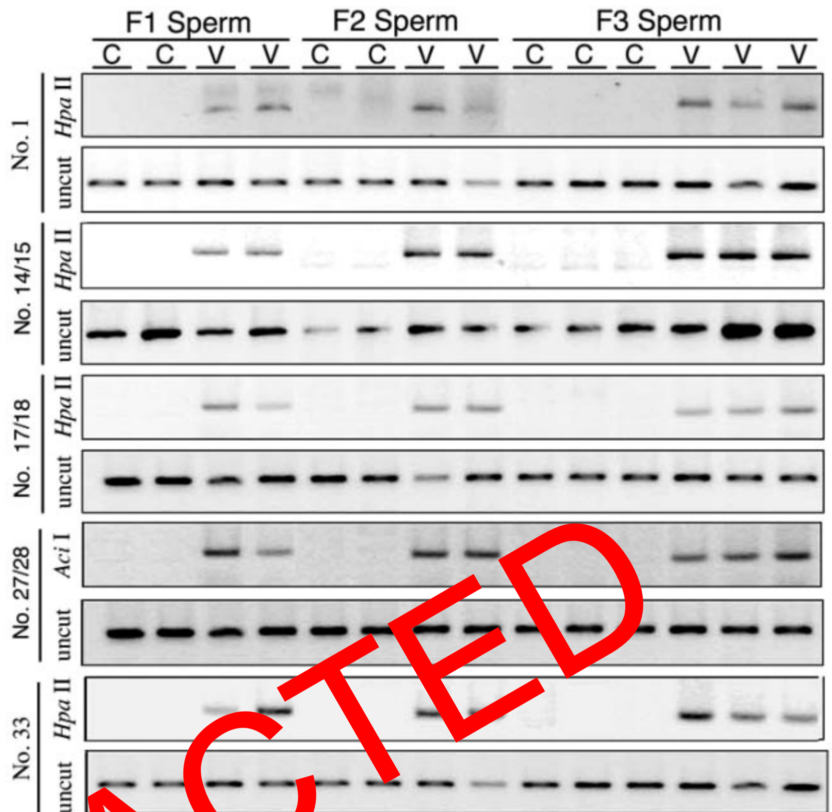

FIG. 5. DNA methylation states in epididymal sperm samples from control (C) and vinclozolin (V) F1, F2, and F3 generations using methylation sensitive (*Hpa*II or *Aci*I) restriction enzyme PCR analysis. Candidates 1, 14/15, 17/18, 27/28, and 33 digests are presented. The bands presented are representative of sperm DNA from different animals from each generation. PCR primers are provided in supplemental Table S1.

in the male germline identified develop an imprinted-like characteristic and appear transgenerational.

The consequence of the epigenetic changes in the male germline induced by vinclozolin and present in subsequent generations was investigated by examining the gene expression of the genes identified to be associated with the DNA methylation sites identified. E16 testes were isolated from control and vinclozolin F1 and F2 generation animals followed by RNA isolation and microarray analysis. The genes that were found to be expressed in the embryonic testis are shown in Fig. 6, and all others were either not present on the microarray chips or were below the detection limit demonstrating a lack of expression in the E16 testis. The gene expression profile between wild-type E13, E14, and E16 testis is presented (Fig. 6A). The E16 F1 and F2 controls are compared with the E16 F1 and F2 vinclozolin generation expression. A number of the genes had reduced expression in the vinclozolin F1 and F2 generation E16 testis including *ankyrin 28*, *Ncstn*, *Rab12*, and *Lrrn6a* (Fig. 6). The genes that had increased expression in the vinclozolin F1 and F2 generation E16 testis compared with control were *Fadd*, *Pbm1b*, *snRP1c*, and *Waspip* (Fig. 6A). Therefore, the effects of the hypermethylation of the genes shown in Figs. 3 and 4 caused both increases and decreases in gene expression in the developing embryonic testis. Whether the changes in gene expression continue to be transgenerational requires future analysis of the F3 generation. The role of these genes in testis development has not been previously investigated.

The expression of one of the known genes nearest an altered methylation site identified, candidate 1 and 21/32 (*NCAM1*), is primarily brain specific such that an analysis of gene expression for *NCAM1* in the brain was performed. Brains from adult control and vinclozolin F3 generation males were isolated and RNA prepared. Four control and five vinclozolin F3 generation animals were analyzed separately. A semiquantitative PCR was developed for *NCAM1* and demonstrated the *NCAM1* mRNA levels were significantly reduced in vinclozolin generation animals, compared with controls (Fig. 6B). An internal constitutively expressed gene, ribosomal protein L19, demonstrated the integrity of the RNA and equal loading with control. This decreased expression of *NCAM1* was quantitated and a more than 10-fold reduction in expression was observed between control and vinclozolin F3 generation males (Fig. 6C). Combined observations suggest the epigenetic alterations identified in the male germline appear to associate with alterations in gene expression in developing organs. More thorough analysis of the complete effects on gene expression in a variety of different organs is now required.

The specific genes associated with the epigenetic changes identified were examined for potential functional relationships. For the genes with known functions or homologs a Pathway Assist bioinformatics program was used to identify functional links between the various genes (supplemental Fig. S4). A number of cellular functions and processes (*e.g.* proliferation, maturation, motility) are affected by the various genes, but no major clustering was observed among the different genes (supplemental Fig. S4).

A number of the genes associated with the methylation changes identified have a relationship with various disease states (Table 2). Interestingly, all of these genes have been shown to have an epigenetic component to the dis-

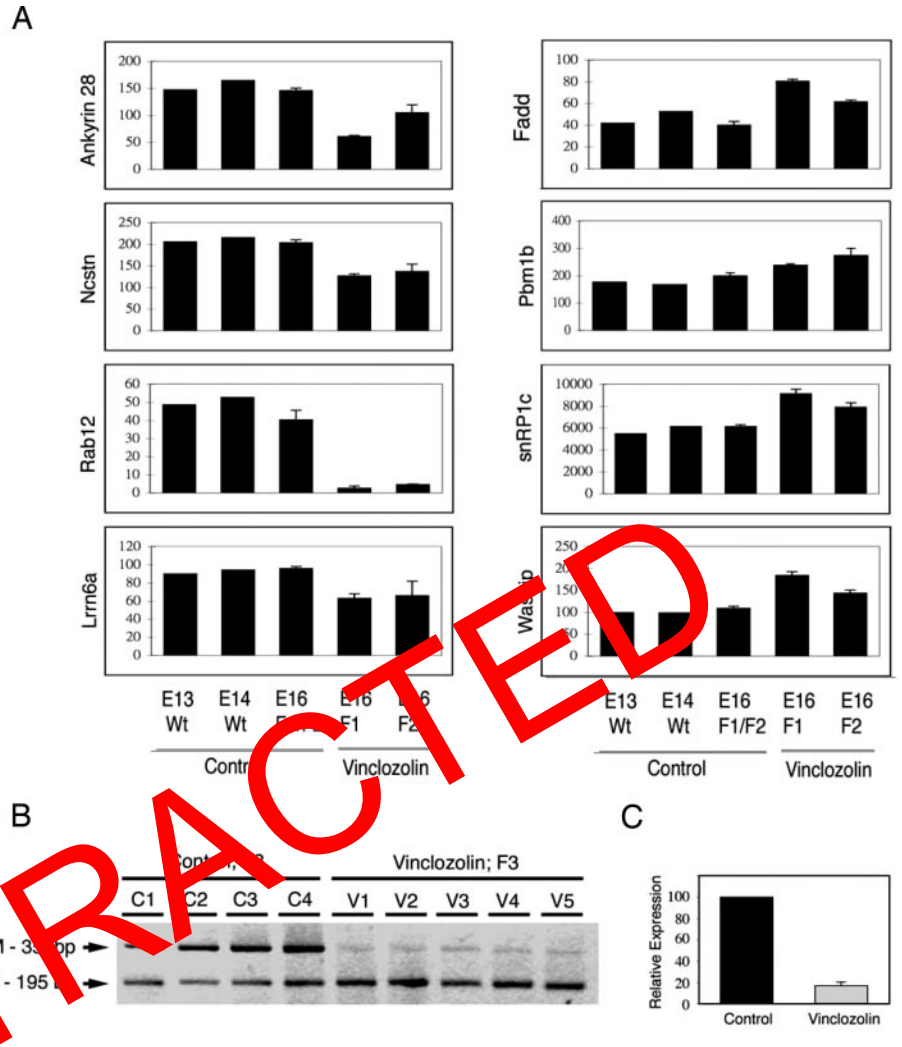

FIG. 6. A, Microarray gene expression analysis of E13, E14, and E16 testis from control and vinclozolin F1 and F2 generations and wild-type (Wt) controls. Relative hybridization signal, mean ± SEM is presented. Two different experiments, microarray chips, and RNA samples were used for each data point. B, Adult brain gene expression for *NCAM1* in control and vinclozolin F3 generation individual animals using a semiquantitative PCR, compared with a constitutively expressed gene L19. The PCR analysis presents the 351-bp *NCAM1* product and 195-bp L19 with each lane representing a different animal. C, The quantitation of relative brain expression levels of NCAM1 for the control and vinclozolin data, mean ± SEM.

ease and/or gene identified (Table 2). Therefore, alterations in the epigenetic (DNA methylation) and/or expression of these genes is associated with a number of disease states. In addition to these correlations to previous literature, the vinclozolin generation animals develop a variety of disease states (2). Previously we have demonstrated that vinclozolin (F1–F4) generation animals between 6 and 12 months of age develop a variety of disease states or abnormalities (Table 3) (1, 2). Therefore, the vinclozolin generational animals used develop a variety of diseases (2) and have the altered epigenetic programming of the male germline. Combined observations indicate that the epigenetic alterations in the germline DNA and associated genes identified in the current study are associated with a number of disease states (Tables 2 and 3). The causal relationship of these epigenetic alterations remains to be elucidated.

Discussion

Embryonic or early postnatal exposure to environmental compounds such as endocrine disruptors have been shown to promote abnormal development and disease in a variety of species, including humans (31–34). Although a large number of environmental factors, such as smoking and caloric restriction (24, 35), and synthetic compounds ranging from plastics to pesticides (22, 23) can promote adult-onset disease and abnormalities, the mechanisms of action of these factors are largely unknown. The observations made in the current study demonstrate that exposure to vinclozolin during embryonic gonadal sex determination promotes an epigenetic imprinting (DNA methylation program) of the male germline (*i.e.* sperm) that is associated with the transgenerational disease phenotype observed.

Methylation of DNA is a critical epigenetic factor in the regulation of gene expression (36), mammalian development (37), and disease (*e.g.* tumorgenesis) (38–40). Specific DNA methylation patterns can have a role in regulating the ability of promoter regions of genes to respond to transcriptional activator complexes and/or influence chromatin structures that influence gene expression (41). Although the vast majority of genomic DNA methylation is reset after fertilization (8), a small subset of imprinted genes maintain a defined DNA methylation pattern that is transmitted through the male or female germline, resulting in allelic expression differences (42–44). Imprinted genes are monoallelically expressed in a parent-of-origin manner and recently a bioinformatics approach has identified as

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TABLE 2. Summaries of related diseases associated with the candidate genes

Candidate no.	Gene symbol	Related disease	Epigenetic link	Ref.
1	NCAM1	Alzheimer Synovial sarcoma Schizophrenia Mutant-allele-specific amplification (MASA) syndrome Neural tube defects Various tumor	Yes	53–58
2, 3, 24	*Lrpprc*	Nociceptor	Yes	59
4, 19	*Cacna1e*	Hyperglycemia Arrhythmia Familial hypokalemic periodic paralysis Male infertility	Yes	62–65
7	*Ankrd28*	Bare lymphocyte syndrome	Yes	89
8	*Fadd*	Cardiomycyte defect Neimann-Pick type C disease	Yes	60, 61
13	*Nfix*	Hipocampal commissure detect	Yes	90
16	*Optineurin*	Open angle glaucoma	Yes	66, 67
17, 18	*Nicastrin*	Alzheimer	Yes	88, 91, 92
21, 32	*Phosphoglycerate mutase*	Neuromuscular disorder	Yes	93
23	*U1 snRNP*	Systemic autoimmune diseases	Yes	94, 95
25	*Mup4*	Renal cysts	Yes	96, 97
29	*Runx1*	Acute myeloid leukemia Rheumatoid arthritis Myelodysplastic syndromes Spenomegaly	Yes	68
31	*Waspip*	Wiskott-Aldrich syndrome	Yes	72
33	*Six3*	Holoprosencephaly Extraskeletal myxoid chondrosarcomas	Yes	73–75, 98

many as 600 candidate imprinted genes in the mouse genome (45). Although the specific monoallelic expression pattern was not directly determined for all the genes identified in the current study, the involvement of the paternal allele was determined by examining sperm from multiple generations. None of the rat genes identified or mouse homologs were on the 600 candidate mouse list reported (45), but species differences could be an issue. One of the genes identified Runx1 was on the nonimprinted gene list used (45). The current study presents the novel observation that an environmental factor can reprogram the germline to promote the presence of new imprinted-like genes/DNA sequences. These new imprinted-like genes/DNA sequences acquire an altered DNA methylation pattern that is transferred through the male germline (*i.e.* paternal allele) to subsequent generations (F1-F3). The current observations suggest an epigenetic reprogramming of the germline is possible. The ability of an environmental factor to alter the transgenerational epigenetic background of an individual and all subsequent progeny has significant impacts on disease etiology.

The caudal epididymal sperm from control and vinclozolin F1-F3 generation males were collected to elucidate potential differences in DNA methylation. The procedure used in the current study involved a MSRE analysis followed by PCR to identify alterations in DNA methylation. This analysis provided 25 different candidate DNA sequences with potential altered methylation. Other procedures involving methylation binding proteins and microarrays (46) have recently been developed. Unfortunately, neither the rat genome or rat gene promoter microarrays are currently available, such that these procedures were not possible in the current study. These procedures may have provided a greater number of candidate sequences, such that the 25 identified should be considered an underestimate of the total potential epigenetic changes possible. Analysis of the 25 candidates demonstrated 15 had confirmed changes in DNA methylation patterns and that these patterns were transgenerational (*i.e.* imprinted-like). The 10 genes/DNA sequences that were not confirmed are speculated to be imprinted-like due to the procedure involved in their isolation; however, further analysis of flanking DNA sequences is required to identify the specific methylation changes. A number of the altered DNA methylation sites were in noncoding regions distal to the nearest genes. The speculation is that these sites may be involved in chromatin structural changes (*e.g.* positioning) that influence the distal genes. The *NCAM1* expression is an example of this potential regulation. Further analysis of these DNA methylation sites is now required to determine their functional roles.

TABLE 3. Vinclozolin inducted (F1–F4) transgenerational disease states

Disease or abnormality	Prevalence (%)
Testis defect	30
Prostate disease	50
Kidney disease	40
Tumor development (*e.g.* breast)	17
Immune abnormalities	27

Summary of disease prevalence in vinclozolin F1–F4 generation animals 6–12 months of age, as previously described (2).

The expression analysis of several of the genes associated with the candidate methylation sites demonstrated altered gene expression in the F1 and F2 vinclozolin generations embryonic testis. Analysis of one brain-specific gene, *NCAM1*, demonstrated a dramatic reduction in *NCAM1* expression in the adult brain of vinclozolin generation males. Therefore, the impact of the transgenerational epigenetic imprinting on the male germline is an influence on the gene

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expression of relevant genes in specific organs. Further investigation of the effects of the transgenerational epigenetic phenotype on the transcriptomes of different organs is now required.

The genes associated with the altered DNA methylation sites range from cell adhesion molecules, ion channels, signaling factors such as phosphatases and GTP binding proteins, transcription factors, translational control factors, and membrane proteins (Table 1). A bioinformatics pathway analysis demonstrated no major cluster or direct relationships between the various genes, but cellular processes such as proliferation, maturation (*i.e.* differentiation), motility, and assembly (*i.e.* structural) are affected by the genes identified. All the imprinted-like DNA sequences identified were hypermethylated, compared with control generation sperm DNA. This hypermethylation suggests potential mechanisms involved in the imprinting mechanism, but the molecular aspects of imprinting and the role of gonadal sex determination remain to be elucidated. Several factors may contribute to the molecular mechanisms of the altered methylation (*i.e.* imprinting) observed. The paternal transmission of the phenotype (1, 2) transgenerationally suggests a role of the Y chromosome and/or paternal imprinted genes. Negligible information is available on the rat Y chromosome such that future studies will be required to examine the influence Y chromosome epigenetics may have on the phenotype and altered methylation of the germline. The ability of paternal imprinting to influence the maternal allele also needs to be considered. Because the maternal allele appears to be ignored and/or modified in the paternal transmission (trans-generationally, unique imprinting mechanisms are suggested. The potential involvement of paramutation mechanisms also need to be considered in allelic communication (47). Independent of the mechanism involved, the current study demonstrates the ability to alter the germline epigenome that then can transgenerationally influence disease development. Future studies will need to be focused on this mechanism.

A number of human diseases are the result of abnormal epigenetic (*i.e.* DNA methylation) programming including Angelman's, Beckwith-Wiedemann, and Prader-Willi syndromes (48–50). Alterations in the DNA methylation patterns of imprinted genes have also been shown to promote the development of disease (45). Potential epigenetic abnormalities in children from *in vitro* fertilization (*i.e.* intracytoplasmic sperm injection) have been identified (51). A study of monozygotic twins suggested environmental epigenetic effects on disease (52). Therefore, numerous studies and clinical conditions suggest epigenetics may be a critical factor in disease etiology.

The previous studies have demonstrated that an environmental factor (endocrine disruptors) can epigenetically reprogram the germline to promote transgenerational disease (1, 2). The current study identified the induction of imprinted-like genes/DNA sequences in the male germline transgenerationally. A number of the associated genes have been shown to be correlated to known disease (Table 2) including Alzheimers (*Nicastrin* and *NCAM1*) (53–58), polymodal nociceptor (*Lrppc*) (59), bare lymphocyte syndrome (*Ankrd 28*) (60, 61), hyperglycemia (*cacnale*) (62–65), open angle glaucoma (*optineurin*) (66, 67), acute myloid leukemia (*Runx1*) (68–71), Wiskott-Aldrich syndrome (*Waspip*) (72), and holoprogencephaly (*Six3*) (73–75). Therefore, a number of the epigenetic changes identified were associated with genes previously shown to have epigenetic links to human disease. As an example, NCAM1 is critical in neural tube and brain development, and abnormalities in NCAM1 cause a number of brain diseases and abnormalities, including Alzheimer's (53–58). Observations demonstrate a dramatic reduction in *NCAM* expression transgenerationally in the vinclozolin generation adult males. In addition, nicastrin interacts with the γ-secretase complex and is critical for normal secretase membrane function (76). The inhibition of nicastrin alters γ-secretase cleavage activity and has been linked as a factor in Alzheimer's disease (77). Epidemiological studies have indicated that Alzheimer's has a potential paternal transmission and paternal age is a risk factor for this disease (78). Combined observations suggest that an environmental factor can induce an imprinted-like epigenetic change in two different genes associated with brain disease and Alzheimer's that transmit their epigenetics through the male germline (paternal allele) as a potential factor in the disease. Although the current study identifies a number of imprinted-like genes/DNA sequences associated with disease, future studies are required to determine the cause and/or effect of these alterations in the epigenome on the disease etiology.

Previously we demonstrated that embryonic exposure to vinclozolin during sex determination promotes the development of adult disease transgenerationally (1, 2). These transgenerational diseases include male fertility abnormalities, tumor development, kidney disease, prostate disease, and immune abnormalities (2). The vinclozolin generation animals used in the current study to identify the epigenetic imprinting in the sperm DNA develop disease states at approximately an 85% frequency (2). Therefore, the imprinted-like genes/DNA sequences identified are associated with these disease states, but a direct causal link or correlation remains to be elucidated. Further investigation of these imprinted-like genes/DNA sequences and correlation to various diseases is speculated to identify novel epigenetic diagnostic and therapeutic targets not previously considered.

The concentration of vinclozolin used in the current study is higher than anticipated in the environment. For vinclozolin the lowest observed adverse effect level recommended for humans is 11 mg/kg·d, but doses at the 1 mg/kg·d have biological effects (79). For rats the lowest observed adverse effect level has been reported as 25 mg/kg·d (80), whereas the no observable and adverse effect level is reported as 6 mg/kg·d (81). The environmental levels of vinclozolin have not been rigorously determined, such that no conclusions regarding the toxicology of this compound can currently be made. Toxicology studies to determine whether environmental levels of the compound can induce these disease states are now needed. The mechanism of vinclozolin actions could involve androgen receptor-mediated events and/or toxicity. The androgen receptor has been shown to be present in the embryonic testis at the time of gonadal sex determination in the germ cells, Sertoli cells, and precursor peritubular cells (82, 83). Although the embryonic gonadal steroid production is minimal at this time, androstenedione is produced by the fetal adrenal. Therefore, endocrine disruptors

have the capacity to influence embryonic androgen receptor actions (84, 85). Alternatively, vinclozolin actions could involve toxicologic actions on the developing gonad to subsequently influence germ cell development (81, 86, 87). An experiment comparing the actions of a defined antiandrogenic compound such as flutamide with those of vinclozolin is in progress. Further studies are needed to elucidate the endocrine *vs.* toxicologic actions of vinclozolin on the embryonic testis.

Although epigenetics is associated with a number of diseases and abnormalities, the current major paradigm for disease etiology involves classic genetics and DNA sequence mutations as a major factor. Clearly regional differences in disease frequencies and environmental influences have suggested additional factors in the development of disease. The observations presented in the current study demonstrate that environmental compounds (endocrine disruptors) can induce a reprogramming in the epigenetic imprinting of the germline. The ability of an environmental factor to promote a permanent epigenetic change in the germline suggests an epigenetic component in disease etiology and a molecular mechanism for the ability of environmental factors to influence disease. This transgenerational epigenetic mutagenesis involves the ability of an environmental factor (*e.g.* endocrine disruptor) to influence embryonic development at the time of sex determination to epigenetically reprogram the germline through the induction of new imprinted-like genes/DNA sequences that then lead to epigenetic alterations in gene expression to promote disease states transgenerationally. Further analysis of this epigenetic disease etiology will provide novel epigenetic diagnostics and therapeutic targets to advance disease therapy. In addition to disease etiology this transgenerational epigenetic mutagenesis also impacts basic developmental biology and broader areas of biology such as evolution.

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