Zfx mutation results in small animal size and reduced germ cell number in male and female mice

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SUMMARY

The zinc-finger proteins ZFX and ZFY, encoded by genes on the mammalian X and Y chromosomes, have been speculated to function in sex differentiation, spermatogenesis, and Turner syndrome. We derived *Zfx* mutant mice by targeted mutagenesis. Mutant mice (both males and females) were smaller, less viable, and had fewer germ cells than wild-type mice, features also found in human females with an XO karyotype (Turner syndrome). Mutant XY animals were fully masculinized, with testes and male genitalia, and were fertile, but sperm counts were reduced by one half. Homozygous mutant XX animals were fully feminized, with ovaries and female genitalia, but showed a shortage of oocytes resulting in diminished fertility and shortened reproductive lifespan, as in premature ovarian failure in humans. The number of primordial germ cells was reduced in both XX and XY mutant animals at embryonic day 11.5, prior to gonadal sex differentiation. *Zfx* mutant animals exhibited a growth deficit evident at embryonic day 12.5, which persisted throughout postnatal life and was not complemented by the *Zfy* genes. These phenotypes provide the first direct evidence for a role of *Zfx* in growth and reproductive development.

Key words: ZFX, zinc-finger protein, ovarian failure, embryonic growth, germ cells, sex determination

INTRODUCTION

Since the discovery of *ZFX* and *ZFY* in humans, investigators have sought to determine the roles these genes might play in mammalian development. The two human genes, located in otherwise nonhomologous regions of the X and Y chromosomes, encode similar but nonidentical proteins (Page et al., 1987; Schneider-Gädicke et al., 1989b). The human ZFX and ZFY proteins – and their homologs in mice and other vertebrates – have a simple two-domain structure (Ashworth et al., 1989; Lau et al., 1989; Mardon et al., 1989b; Schneider-Gädicke et al., 1989a; Mardon et al., 1990; Palmer et al., 1990). The carboxy-terminal half is an uninterrupted string of 13 Cys-Cys/His-His zinc fingers. This ZFX/Y domain consists of alternating fingers of two types, quite unlike other zinc-finger proteins (Page et al., 1987; Mardon and Page, 1989b). The amino-terminal halves of the ZFX/Y proteins are highly acidic and will activate transcription in yeast when fused to the DNA-binding domain of GAL4 (Mardon et al., 1990). Nestled between these two large domains is a highly conserved run of basic amino acids that may serve as a nuclear localization signal. The combination of an acidic activating domain, a nuclear localization motif, and a DNA-binding motif suggests that the ZFX/Y proteins may function as sequence-specific transcription activators.

Studies of human XX males and XY females localized *ZFY* to the sex determining region of the human Y chromosome (Page et al., 1987). Although a central role in sex determination was ruled out by the report of rare human XX males who carried the nearby *SRY* gene but lacked *ZFY* (Palmer et al., 1989), speculation has continued that the ZFX/Y proteins may function in sex differentiation, or in spermatogenesis (Mardon and Page, 1989b; Nagamine et al., 1990; Koopman et al., 1991). Other investigators have proposed that the *ZFX/Y* genes play critical roles in the development of phenotypic features observed in humans with monosomy X (Burgoyne, 1989; Ashworth et al., 1991; Ogata et al., 1993). All of the above hypotheses about the organismal function of the ZFX/Y proteins remained fundamentally untested prior to the present study.

Genetic studies might clarify the role of the ZFX/Y proteins in mammalian development. Since we had no solid *a priori* insights about organismal function, we chose a reverse genetic strategy, mutagenizing a mouse homolog and noting the organismal effects. This strategy required that we choose an initial target from among the four *ZFX/Y* homologs in the mouse: *Zfx*, on the X chromosome; *Zfy1* and *Zfy2*, on the Y chromosome; and *Zfa*, a *Zfx*-derived processed gene on chromosome 10 (Page et al., 1987; Mardon et al., 1989a; Mitchell et al., 1989; Nagamine et al., 1989; Ashworth et al., 1990; Mardon et al., 1990; Page et al., 1990).

We chose *Zfx* for two reasons. First, *Zfx* is transcribed in virtually all male and female tissues and developmental stages examined; transcription of the mouse *Zfy* and *Zfa* genes is much more restricted (Koopman et al., 1989; Mardon et al., 1989a; Ashworth et al., 1990; Mardon et al., 1990; Nagamine et al., 1990; Zambrowicz et al., 1994). Second, among the four mouse proteins, ZFX appears to be under the most intense selective pressure. The amino acid sequence of the mouse ZFX protein is highly similar to that of human ZFX, human ZFY, and even chicken ZFB. The mouse ZFA, ZFY1, and ZFY2 proteins appear to be much less conserved. Among the four mouse proteins, ZFX is probably most representative of the single common ancestral protein from which evolved the family of ZFX/Y proteins present in a wide array of mammals and other vertebrates.

As we report here, the phenotypes observed in *Zfx* mutant animals provide the first direct evidence that the ZFX/Y proteins play critical roles in embryonic growth and germ cell development.

MATERIALS AND METHODS

Gene targeting construct

Lambda phage clone D3-6, containing a 15-kb portion of the mouse *Zfx* gene, was previously isolated from a 129 genomic library (Luoh and Page, 1994). The D3-6 insert was ligated into the *Xho*I site of pBluescript. A 2-kb fragment containing *Zfx* exons 7 through 11 was deleted by digestion with *Bss*HII and *Nco*I. Ligation of adaptors containing an *Xho*I site to the *Bss*HII and *Nco*I ends allowed us to insert the *Pgk-neo*^{r}-poly(A) cassette from pKJ-1 (Li et al., 1992) at the newly added *Xho*I site. The particular plasmid used in subsequent procedures contained two tandemly repeated copies of the *Pgk-neo*^{r}-poly(A) cassette (Fig. 1). The construct retained 2 kb of *Zfx*-homologous sequence 5′ of the cassette and 11 kb of *Zfx*-homologous sequence 3′ of the cassette.

ES cell electroporation and selection

The 129-derived ES cell line J1 was cultured as previously described (Li et al., 1992) except that embryonic fibroblasts treated with mitomycin-c (Sigma, St Louis, Missouri) served as feeder layers. ES cells were trypsinized, resuspended at a density of 1 million cells/ml in phosphate-buffered saline without calcium or magnesium (PBS), and electroporated in the presence of 25 µg/ml of linearized targeting construct DNA at 1250 V/cM, 250 µF, with a pulse length of 1.2 to 1.4 milliseconds, using an ECM 600 electroporator (BTX, San Diego, California). Ten minutes after electroporation, ES cells were plated on a feeder layer of G418-resistant embryonic fibroblast cells. Twenty-four hours later, selection with G418 (Gibco-BRL, Gaithersburg MD) at 250 µg/ml (active form) was begun. G418-resistant colonies were transferred to 96-well dishes for continued selection. After reaching confluency (typically at 2-3 days), clones were transferred to 24-well dishes for further expansion. When confluent, half of the cells in each well were frozen while the other half were plated in 12-well dishes without feeder cells for DNA isolation and Southern analysis.

Generation of *Zfx* mutant mice

Two independent targeted clones, ES211 and ES212, were injected into BALB/c or C57BL/6J blastocysts and chimeras were generated, as previously described (Li et al., 1992). Chimeric males were mated with BALB/c, C57BL/6J, or 129/SvJae females. (BALB/c and 129/SvJae mice were bred and maintained at Whitehead Institute. C57BL/6J mice were obtained from Jackson Laboratory.) Because *Zfx* is X-linked, all F$_1$ animals carrying the mutant allele were heterozy-

gous (+/−) females. These animals were backcrossed to BALB/c, C57BL/6J, or 129/SvJae males to obtain hemizygous (−/Y) N2 males and heterozygous N2 females. All experiments described in this report were conducted using N2 animals or their random-bred descendants.

Genotyping by Southern blotting or PCR

We tested cells and tissues for the presence of wild-type and mutant *Zfx* alleles, and for the Y chromosome, by Southern blotting or PCR. DNAs were prepared from ES cells, from tail biopsies of weanlings, or from tail, limb, or yolk-sac fragments of embryonic mice as previously described (Laird et al., 1991).

For genotyping by Southern analysis, DNAs were digested with restriction endonucleases, subjected to agarose electrophoresis, transferred to nylon membrane, and hybridized with radiolabeled probes as described (Mardon et al., 1990). Hybridization probes were as follows: (i) probe A, from the *Zfx* gene, a 0.3-kb *Sal*I-*Stu*I fragment purified from plasmid pDP1529, a subclone of a 1.9-kb *Sal*I-*Eco*RI fragment from genomic phage BALB/c-10 (Luoh and Page, 1994); (ii) probe B, also from the *Zfx* gene, a 0.9-kb *Eco*RI fragment purified from plasmid pDP1530, a subclone of a 6.1-kb *Spe*I fragment from genomic cosmid cEMS-163 (Luoh and Page, 1994); and (iii) from the Y chromosome, a 0.8-kb *Hin*dIII-*Sal*I fragment of plasmid PY2, which hybridizes to a mouse Y-specific repetitive sequence (Lamar et al., 1984).

PCR genotyping was carried out using the following oligonucleotide pairs: (i) for the wild-type *Zfx* allele, ATGCACACTAG-CATTGCCTG (intron 10) and TTCTGATGGAATTGAAAACCG (exon 10), yielding a product of 198 bp; (ii) for the mutant allele, *neo*^{r} primers AGACAATCGGCTGCTCTGAT and ATACTTTCTCG-GCAGGAGCA, yielding a product of 261 bp; and (iii) for the Y chromosome, *Zfy1* primers AAGATAAGCTTACATAATCACATGGA and CCTATGAAATCCTTTGCTGCACATGT, yielding a product of 700 bp. PCR and electrophoresis conditions were as described previously (Reijo et al., 1995), except that PCR annealing was at 55°C.

Histological analysis

Whole embryos or tissues were fixed in either 10% buffered formalin or Bouin's solution, dehydrated in graded alcohol and xylene, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Total oocyte numbers in 4-day-old and adult mice were experimentally estimated using serial sections of paraffin-embedded, hematoxylin/eosin-stained ovaries. Ovaries of 4-day-old and adult mice were sectioned at thicknesses of 3.5 and 7 µm, respectively. In the case of 4-day-old mice, we counted all oocyte nuclei in every 20th section. In the case of adult mice, we counted oocyte nuclei in antral (type 3b-5b) and preantral follicles (type 6-8) in every tenth section (Pedersen et al., 1968). Oocytes in primordial follicles (type 1-3a) could not be enumerated reliably and were not counted. The numbers of oocyte nuclei in all sections were summed and multiplied by the section interval (10 or 20) to arrive at a crude estimate of oocyte number per ovary. To correct for the effect of nuclear diameter, we multiplied the crude estimate by the ratio of the section thickness to the sum of the section thickness plus the mean nuclear diameter (Abercrombie, 1946). Based on our measurements, we estimated mean nuclear diameters of 10, 12, and 16 µm for 4-day-old, preantral follicle, and antral follicle oocytes, respectively.

To assess skeletal development, embryos were stained with alizarin red S and alcian blue 8GS as described by McLeod (1980). Embryos were staged as described by Kaufman (1992).

Estimation of primordial germ cell numbers

Posterior portions of embryos (limb bud stage 3-5; Hogan et al., 1994) were collected at (embryonic day) E11.5, placed in ice-cold 80% ethanol, stored at 4°C, and then cleared in two changes of chloroform before being embedded in paraffin and serially sectioned at a thickness of 7 µm. After counterstaining with eosin, we counted germ

cells, identified by alkaline phosphatase expression (Buehr et al., 1993), in every fifth section. The germ cells counted included those present in the genital ridges as well as those still migrating through the dorsal mesentery. We estimated the total number of germ cells in each embryo using the numerical methods described above for oocytes, in this case estimating a mean germ cell diameter of 9 μm. Anterior portions of the same embryos were used for genotyping.

RESULTS

Generating a mutation in *Zfx*

We set out to delete exons encoding a nuclear localization signal and much of the acidic domain of *Zfx* via homologous recombination in mouse embryonic stem (ES) cells. We have previously shown that the mouse *Zfx* gene spans 50 kb and contains at least 11 exons, the first four of which are not translated (Luoh and Page, 1994). Exons 5 through 10 encode the acidic domain, and exon 10 also encodes a nuclear localization motif (Mardon et al., 1990). Exon 11 encodes all 13 zinc fingers and contains the 3′ untranslated sequences. In our gene-targeting construct, *Zfx* exons 7 through 10 were replaced by a neomycin resistance gene (*neo^r*) duplicated in tandem and under the control of a PGK promoter (Fig. 1A). Successful targeting of the endogenous *Zfx* gene using this construct will result in deletion of half of the protein's acidic domain and a nuclear localization signal. Despite repeated efforts, our laboratory has been unsuccessful in generating antisera of sufficient quality to determine the presence or absence of an altered ZFX protein in mutant cells – perhaps because of the high degree of conservation of ZFX amino acid sequence among mammals. The linearized construct was transfected into J1 embryonic stem (ES) cells (Li et al., 1992). These ES cells derive from a normal XY male mouse and contain a single copy of the *Zfx* gene. By Southern blot analysis of DNAs from 225 resulting G418-resistant clones, we identified five clones that had undergone the desired homologous recombination events (and whose genotype will hereafter be referred to as '−/Y'; Fig. 1B).

To generate chimeric mice harboring the *Zfx* mutation, cells of two independent −/Y ES lines, ES211 and ES212, were injected into wild-type blastocysts (BALB/c or C57BL/6J) in vitro and transplanted into foster mothers. Efficient germline transmission of the *Zfx* mutation was observed when chimeric males derived from either ES211 or ES212 were mated with wild-type (+/+) BALB/c or C57BL/6J females. All offspring inheriting the *Zfx* mutation were, as expected, heterozygous (+/−) females.

Diminished neonatal viability

When the +/− females were mated with wild-type (+/Y) males (BALB/c or C57BL/6J), a substantial minority of the resulting progeny were found dead or dying within 24 hours of birth. The dying pups were small and had no milk in their stomachs; we detected no other gross abnormalities.

Southern blotting studies revealed that the observed neonatal lethality was essentially limited to *Zfx* mutant progeny. One would expect this cross to yield the following four classes of progeny, in equal numbers: +/−, +/+, −/Y and +/Y. However, of 42 dead pups collected, 34 were −/Y, seven were +/−, and only one was wild-type (+/+ or +/Y; presence or absence of Y

not determined conclusively). Conversely, among progeny who survived to the age of weaning (3-4 weeks), the representation of mutant classes was far below Mendelian expectations. It appears that of the −/Y and +/− progeny, roughly one tenth and one half, respectively, survived to weaning (Table 1, experiments 1 to 3).

These effects on viability were seen with animals derived from two independent *Zfx* mutant ES cell lines (ES211 and ES212) and thus are very likely the direct consequence of the *Zfx* mutation. In these crosses, the effect of genetic background (C57BL/6J vs. BALB/c) was minimal (Table 1, compare experiments 2 and 3). However, we were unable to propagate the mutation on a pure 129/SvJae strain background, suggesting that important modifiers of mutant viability may exist elsewhere in the genome. Subsequent experiments focused primarily on animals derived from ES211 by breeding to BALB/c.

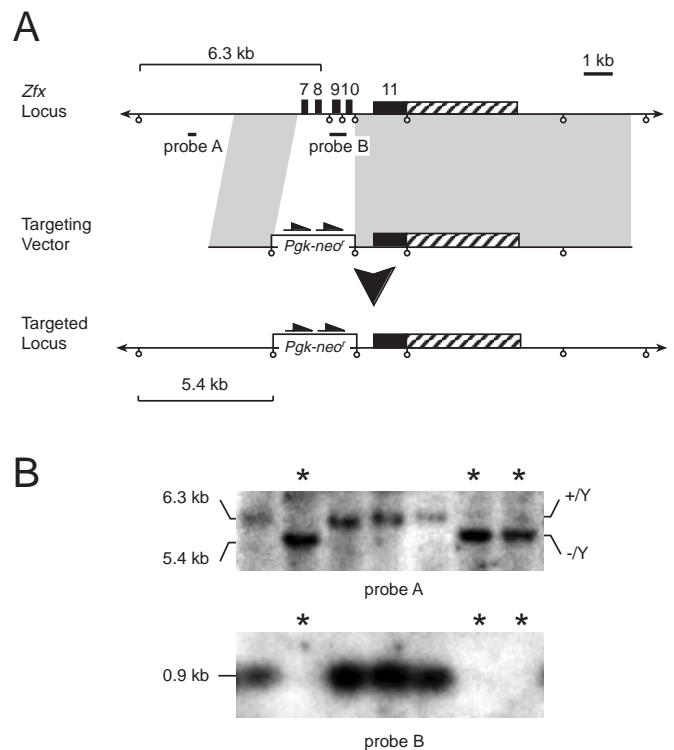Mutant (−/Y) males surviving to adulthood proved to be

Fig. 1. Targeted mutagenesis of *Zfx*. (A) Structure of portion of wild-type gene, targeting construct, and predicted structure of correctly targeted gene. Grey areas indicate regions of homology between *Zfx* and targeting vector. Black boxes represent coding portions of exons 7 through 11. Hatched box represents 3′-untranslated portion of exon 11. Open boxes represent tandemly duplicated *neo^r* genes within construct; arrows indicate their direction of transcription. Size and location of probes A and B, used to detect homologous recombination events, are shown. *Eco*RI sites represented by small circles. (B) Southern blot analysis of *Eco*RI-digested genomic DNAs from mutagenized ES cell clones. Asterisks denote correctly targeted clones. (Top) Probe A hybridizes to 6.3-kb fragment from wild-type X chromosome and 5.4-kb fragment from correctly targeted X chromosome. (Bottom) Probe B hybridizes to 0.9-kb fragment from wild-type X chromosome but has no homolog on correctly targeted X chromosome. Same blot hybridized sequentially with probes A and B.

Table 1. Survival of offspring of +/− x +/Y and +/− x −/Y matings

Experiment	Cross	ES cell line of origin	Background strain	Age at counting	Total number of surviving offspring, by genotype*					$\chi^2 > 7.8$
					+/Y	−/Y	+/+	+/−	−/−	
1	+/− × +/Y	ES211	BALB/c	3-4 wks	90	19	85	59	—	50
2	+/− × +/Y	ES212	BALB/c	3-4 wks	55	4	53	22	—	55
3	+/− × +/Y	ES212	C57BL6	3-4 wks	24	0	23	9	—	14.7
4	+/− × −/Y	ES211	BALB/c	3-4 wks	102	8	—	86	4	158
5	+/− × −/Y	ES211	BALB/c	3-4 wks	50	12	—	40	10	43
6	+/− × −/Y	ES211	BALB/c	E18.5	38	29	—	26	24	—
7	+/− × −/Y	ES211	BALB/c	E15.5	16	6	—	13	15	—
8	+/− × −/Y	ES211	BALB/c	E12.5	13	12	—	10	12	—

*Number of viable animals at 3-4 weeks post partum or present in utero; each experiment represents sum of numerous litters. χ^2 values shown if greater than 7.8, which corresponds to $P<0.05$. Modest differences in survival of mutant animals in experiments 4 and 5 may reflect fact that in experiment 4 (as in experiments 1-3), two or three nursing dams and their litters were housed in a single cage, while in experiment 5, each cage held only one dam and her litter.

fertile. To generate −/− females, we crossed +/− females with −/Y males. If the mutation had no effect on viability, this cross would be expected to yield animals of the following four genotypes, in equal numbers: −/−, +/−, −/Y and +/Y. However, as in the previous cross, there was substantial mortality in the first postnatal day. Among progeny who survived to weaning, the three mutant classes – and especially the −/− and −/Y animals – were underrepresented. Of the −/Y, +/−, and −/− progeny, roughly one tenth, four fifths and one tenth, respectively, survived to weaning (Table 1, experiments 4 and 5).

The poor survival of mutant animals to weaning is evidently due, at least in part, to an inability to weather the neonatal period. However, it remained possible that poor survival in utero also contributed to the marked underrepresentation of mutant animals at weaning. To test this possibility, we harvested litters from matings (+/− × −/Y) at E18.5, 1-2 days before birth would have occurred, or at earlier embryonic stages. Typing of the embryos revealed the presence of wild-type and mutant classes in proportions not significantly different from Mendelian expectations (Table 1, experiments 6 to 8). The viability of *Zfx* mutant embryos is indistinguishable from that of wild-type (+/Y) littermates. We conclude that most if not all premature deaths of *Zfx* mutant animals occur in the neonatal period. We have not yet detected any anatomic or histologic abnormalities that would account for these deaths.

Reduced body mass of embryos and adults

We noticed many small animals among the surviving progeny of both +/− × +/Y and +/− × −/Y crosses. A comparison of body mass and genotype suggested that the small animals were largely if not exclusively −/Y males and −/− females. We therefore charted the growth at weekly intervals of all progeny from several +/− × −/Y matings. We found that, throughout the first six weeks of life, −/Y males were 20-25% smaller than +/Y males and that −/− females were 20-30% smaller than +/− females (Fig. 2).

To determine whether this growth deficit existed before birth, embryos from +/− × −/Y matings were collected and weighed at E12.5, E15.5, and E18.5. At all three time points, −/Y and −/− embryos were roughly 20% smaller than +/Y and +/− littermates (Table 2). We conclude that the reduced body mass of newborn and adult mice lacking an intact *Zfx* gene is the result of a growth disturbance originating prior to E12.5.

A diminished rate of growth in utero might or might not be accompanied by retarded development. To address this question, we examined the timing of skeletal development in mutant and wild-type embryos. We found that the time course of appearance and maturation of ossification centers was very similar in +/Y −/Y, +/−, and −/− animals (data not shown). Thus, as assayed by skeletal development, there appeared to be no significant developmental delay in embryos lacking an intact *Zfx* gene.

Sexual differentiation and fertility

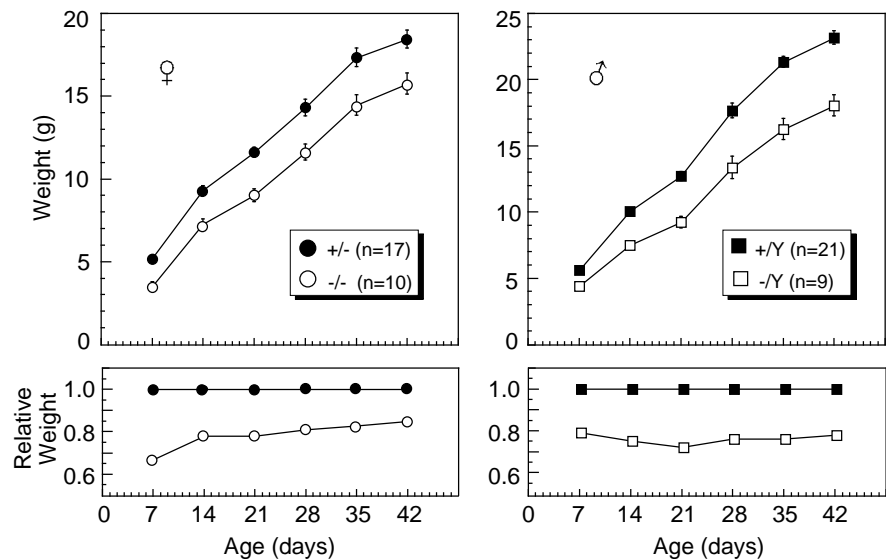We detected no gross abnormalities of sexual differentiation in

Fig. 2. Reduced weight of *Zfx* mutant animals. Offspring of −/Y x +/− mating were weighed once a week beginning at 7 days of age. Upper panels: absolute weights of +/− and −/− females (left) and +/Y and −/Y males (right) expressed as mean ± s.e.m. Lower panels: relative weights of +/− and −/− females (left; normalized to +/−) and +/Y and −/Y males (right; normalized to +/Y).

Table 2. Weights of fetal offspring of +/− x −/Y mating

	Average fetal weight (g), by genotype* (and relative weight)					
	Males			Females		
Age at weighing	+/Y	−/Y	*P* value†	+/−	−/−	*P* value†
E12.5	0.055±0.004 (1.00)	0.042±0.006 (0.76)	<0.06	0.056±0.005 (1.00)	0.043±0.005 (0.77)	<0.07
E15.5	0.36±0.07 (1.00)	0.29±0.04 (0.81)	<0.002	0.36±0.01 (1.00)	0.28±0.01 (0.78)	<0.001
E18.5	1.19±0.04 (1.00)	0.91±0.02 (0.76)	<0.001	1.12±0.04 (1.00)	0.88±0.02 (0.79)	<0.001

*Fetuses are those tabulated in experiments 6-8 in Table 1. Relative weight (in parentheses) calculated by dividing by average weight of +/Y fetuses (in case of males) or of +/− fetuses (in case of females).
†Two-tailed Student's *t*-test.

−/Y, +/−, or −/− animals. Regardless of neonatal viability, −/Y animals were fully masculinized, with anatomically and histologically normal testes, male internal accessory structures, and male external genitalia. Conversely, +/− and −/− animals were fully feminized, with ovaries, female internal accessory structures, and female external genitalia. (As described below, the ovaries of −/− females and testes of −/Y males were small.) Adult −/Y males and +/− and −/− females exhibited normal mating behavior as evidenced by formation of copulatory plugs.

Fertility appeared to be normal in −/Y males and +/− females. Of 35 −/Y animals mated, 33 repeatedly sired litters. Heterozygous females also showed no gross impairment of fertility, as pregnancies resulted from 42 of 61 matings (69%) between +/− females (48 different animals, 7-39 weeks of age) and −/Y males. (Successful mating judged by copulatory plug formation.)

By contrast, the fertility of homozygous mutant females was severely compromised. Pregnancies resulted from only 16 of 51 matings (31%) between −/− females (21 different animals, 7-40 weeks of age) and wild-type males. Whereas numerous +/− females greater than 30 weeks of age continued to bear litters, the oldest −/− female to become pregnant was 25 weeks old, despite continued mating of such animals to an age of 40 weeks. Moreover, −/− females that did become pregnant after mating with +/Y males gave birth to small litters as compared with +/− females mated with −/Y males (3.8±1.9 vs. 6.4±2.1 pups/litter). This striking difference (*P*<0.001) in litter size is not readily accounted for by differential viability of embryos of various genotypes, since the two crosses would be expected to yield many offspring of similar genotype and, in aggregate, litters of comparable viability. Quite apart from any effect of embryonic genotype, it appears that fertility is greatly impaired in females homozygous for the *Zfx* mutation.

Surprisingly, all pups born to −/− females (mated with either +/Y or −/Y males) died within 24 hours of birth. This could not be explained solely on the basis of the pups' genotypes. Fostering experiments in which litters were transferred between −/− dams and +/+ dams demonstrated that the inviability of pups born to −/− females was due to a nursing deficit in −/− females; regardless of genotype, pups nursed by −/− females gained relatively little weight (unpublished results). The developmental and physiologic origins of this nursing deficit remain to be explored.

Oocyte deficit in −/− females

Light microscopic examination of the ovaries of −/− and +/− littermates revealed the cause of the −/− animal's poor fertility: a dramatic reduction in the number of oocytes (Fig. 3). At all ages examined, ovaries of −/− animals were relatively depleted of primary and growing follicles and, as a result, their ovaries were much smaller than those of +/− animals. For example, at 42 weeks of age, when +/− ovaries still displayed a full range of follicular activity (Fig. 3A), −/− ovaries contained no or few oocytes and were largely atrophic and fibrous (Fig. 3B). In some younger −/− females, a qualitatively normal progression of follicular development and ovulatory activity could be observed (Fig. 3D), with maturing follicles, preovulatory follicles, and corpora lutea all present. This is as one would expect given that some young −/− females do become pregnant. Nonetheless, even in these young −/− animals, the number of oocytes was reduced.

To quantify this defect across the reproductive lifespan, we counted oocytes in antral and preantral follicles in the ovaries of −/− and +/− females killed at 8-46 weeks of age. (In wild-type mice, oocyte counts drop precipitously during adult life, necessitating age-matched, time-course studies.) At all ages, we found that the number of follicles per ovary was dramatically reduced in −/− as compared with +/− animals (Fig. 4). Indeed, antral/preantral follicle counts in −/− ovaries fell to negligible levels (less than 100 per ovary) as early as 18 weeks of age, while +/− ovaries showed counts of 200 antral/preantral follicles as late as 46 weeks of age.

Was this oocyte depletion in adult females the result of accelerated loss associated with ovarian cycling (follicular atresia and ovulation), or did it reflect a deficiency in oocytes present prior to the onset of puberty? To answer this question, we determined the total number of oocytes in the ovaries of sexually immature −/− and +/− littermates. At 4-5 days post partum, the number of oocytes in −/− ovaries (304±74 oocytes per ovary; *n*=8) was far lower (*P*<0.002) than that in +/− ovaries (1167±593; *n*=6). Thus, the oocyte deficit in −/− animals existed before the onset of reproductive cycling. Ovarian failure in −/− mice evidently results from an oocyte deficit that exists prior to sexual maturation.

The oocyte count in ovaries of 4-5 day post partum +/− females was lower than that previously reported for normal mice of similar age (Jones et al., 1961; Burgoyne et al., 1985). This suggested that *Zfx* heterozygotes, though fertile, might

have a modest oocyte deficit. This proved to be the case, as we discovered by examining the ovaries of +/− and +/+ littermates from a +/Y × +/− cross. At 4-5 days post partum, the number of oocytes in +/− ovaries (1495±245; n=6) was significantly less (P<0.003) than that in +/+ ovaries (2526±121; n=5). Thus, heterozygous females have an oocyte deficit, though milder than that observed in −/− mice. These findings put the oocyte counts in −/− females, which we had previously compared with +/− females, in starker relief: at 4-5 days of age, −/− females have about one eighth the number of oocytes seen in +/+ animals.

Reduced sperm counts in −/Y males

The oocyte deficits observed in −/− and +/− females led us to investigate more closely whether −/Y males, whose fertility was grossly undisturbed, might also exhibit some abnormality in gamete production. As judged by testicular histology, spermatogenesis appeared to be qualitatively normal in −/Y animals (not shown). However, testicular and epididymal weights, both sensitive measures of spermatogenic activity, were markedly reduced in −/Y as compared to +/Y males, even when corrected for the overall diminished body mass of −/Y males (Table 3). Indeed, the number of sperm present in the epididymides of −/Y mice was less than half that found in +/Y mice. (At greater than 2 million per caput epididymis, the −/Y sperm counts are entirely consistent with fertility.) We conclude that spermatogenesis is qualitatively normal but its output halved in −/Y mice.

Reduced primordial germ cell counts in −/− and −/Y embryos

To explore the embryonic origins of the germ cell deficits observed in mutant males and females, we counted primordial germ cells (PGCs) at E11.5, just prior to gonadal sex differentiation. By E11.5, most PGCs have completed their migration into the genital ridge, but about 5% are still moving through the gut and dorsal mesentery (Tam et al., 1981). At E11.5, *Zfx* mutant and wild-type embryos displayed similar proportions of PGCs remaining outside the genital ridge. We did not observe abnormal migration of PGCs to ectopic sites. As expected, the numbers of PGCs in +/+ and +/Y embryos were nearly identical (Fig. 5). However, the PGC counts in −/− and −/Y embryos were less than half of those in the wild-type embryos (P<0.007 and P<0.001, respectively).

Thus, diminished sperm and oocyte counts in *Zfx* mutant male and female adults are at least partly due to reduced PGC counts prior to gonadal sex differentiation.

DISCUSSION

The pleiotropic consequences of the *Zfx* mutation provide a functional context in which to evaluate the wealth of molecular biologic data that has accumulated about this gene and its homologs in mice, humans, and other vertebrates. We have found that *Zfx* mutant animals exhibit poor neonatal viability, small body size, oocyte deficits that lead to reduced fertility and premature ovarian failure, and reduced sperm counts. Perhaps the pleiotropic effect of the *Zfx* mutation is not surprising, since *Zfx* is reported to be transcribed in every
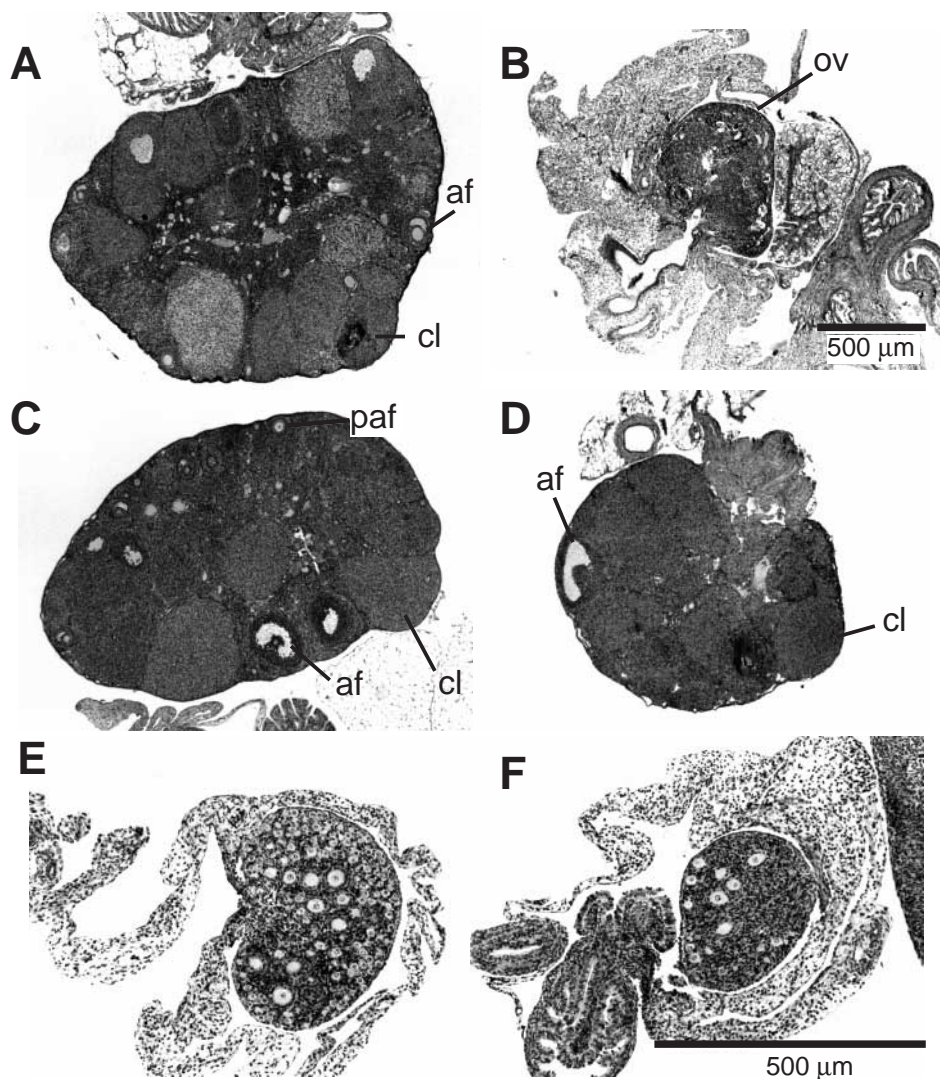

Fig. 3. Ovarian histology in +/− and −/− females. (A,B) 42-week-old animals. (A) +/−; (B) −/−. (C,D) 17-week-old animals. (C) +/−; (D) −/−. (E,F) 4-day-old animals. (E) +/−; (F) −/−. af, antral follicle; paf, preantral follicle; cl, corpus luteum; ov, ovary. Ovaries of +/− mice (A,C) show numerous immature and mature follicles and corpora lutea. Ovaries of −/− mice (B,D) show few or no follicles; corpora lutea are evident at 17 weeks (D), but not at 42 weeks (B). At 4 days of age, number of oocytes is reduced in −/− (F) as compared with +/− (E).
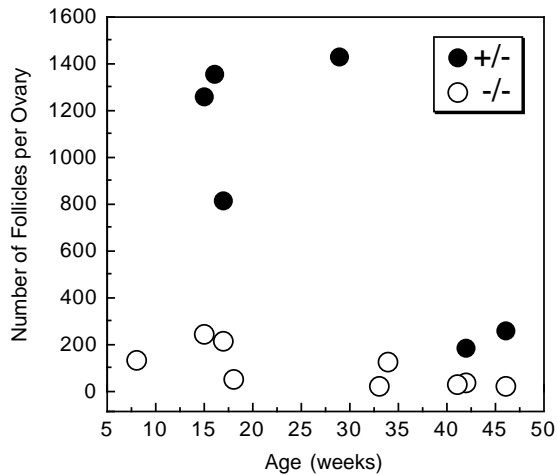
Fig. 4. Total number of follicles (preantral plus antral) per ovary in adult +/− and −/− females. Each point represents mean number per ovary for a single mouse.

embryonic, newborn, and adult mammalian tissue and cell line examined (Schneider-Gädicke et al., 1989b; Mardon et al., 1990; Palmer et al., 1990).

The *Zfx* mutant allele we generated is deleted for four of the gene's seven coding exons, including the highly conserved exon encoding a putative nuclear localization signal (Luoh et al., 1995). Nonetheless, the mutation may not have caused a complete loss of function; it may have resulted in a partial loss of function, or even a gain of function. The latter possibilities arise because of the intron/exon structure of *Zfx*: all coding-region introns are positioned between the first and second nucleotides of a codon triplet, such that any coding exon or combination of coding exons could be spliced out without disrupting the reading frame downstream (Luoh and Page, 1994). Indeed, alternative splicing of human and mouse *Zfx* transcripts is well documented (Schneider-Gädicke et al., 1989a; Ashworth et al., 1990; Luoh and Page, 1994). In the case of our mutant allele, in which exons 7 through 10 (the second through the fifth coding exons) were replaced by a neomycin gene (Fig. 1), it is possible that splicing of exon 6 to exon 11 could produce a truncated ZFX protein retaining the zinc-finger domain, which may harbor a second nuclear localization domain (Taylor-Harris et al., 1995). Antisera with which to test this possibility are not yet available. Thus, the phenotypes observed may reflect a complete or partial loss of function, or a gain of function.

No effect on sex differentiation

Although we found reduced numbers of germ cells in *Zfx* mutant males and females, we detected no abnormalities of the somatic components of the reproductive tract in either sex. We have found no evidence that *Zfx* functions in determining the sexual fate of (1) the somatic portion of the gonad, (2) the internal accessory structures, or (3) the external genitalia. It has previously been suggested that ZFX/Y play an indirect role in gonadal sex determination via an effect on embryonic growth (Mittwoch, 1992). While it is now clear that the *Zfx* mutation slows embryonic growth, this growth defect appears to be of no consequence for gonadal sex differentiation.

Table 3. Gamete production in +/Y and −/Y mice

	Genotype			
	+/Y (n=5)	−/Y (n=6)	Ratio*	P value†
Testicular weight (mg)	240±7	139±8	0.58	<0.0001
Epididymal weight (mg)	52±4	29±2	0.55	0.0009
Sperm/caput (millions)	4.19±0.83	2.05±0.41	0.49	0.0373
Body weight (g)	29.7±1.2	24.1±1.0	0.81	0.0063

*Ratio of −/Y to +/Y.
†Two-tailed Student's t-test.

Embryonic growth and adult size

Several observations in our study indicate that *Zfx* plays a critical role in growth of the early embryo and, thereby, in determining the size of the adult. In mutants of both sexes, growth was disturbed prior to E12.5 and the resulting size deficit persisted throughout fetal development and postnatal life. Males of -/Y genotype were 20-25% smaller than +/Y littermates at all developmental stages examined: E12.5, E15.5, E18.5 and throughout the first 6 weeks of postnatal life. Similarly, −/− females were 20-30% smaller than +/− littermates at these embryonic and postnatal timepoints. Thus, the growth deficit caused by the *Zfx* mutation is readily evident by mid-gestation and is not fully compensated during fetal or postnatal development.

Compared to some other growth factors, *Zfx* seems to influence organismal growth earlier in development. Embryos deficient in growth hormone (by virtue of mutations in the Pit-1 transcription factor or in the receptor for the growth-hormone-releasing hormone) are of normal size, and growth retardation is not evident until about 2 weeks after birth (Camper et al., 1990; Li et al., 1990; Godfrey et al., 1993). Though dramatic postnatal growth retardation has been observed in animals lacking the transcription factor c-*fos*, birth weights were normal (Field et al., 1992; Johnson et al., 1992). While embryonic fibroblasts lacking the transcription factor c-*jun* show reduced growth rates in culture, E12.5 embryos
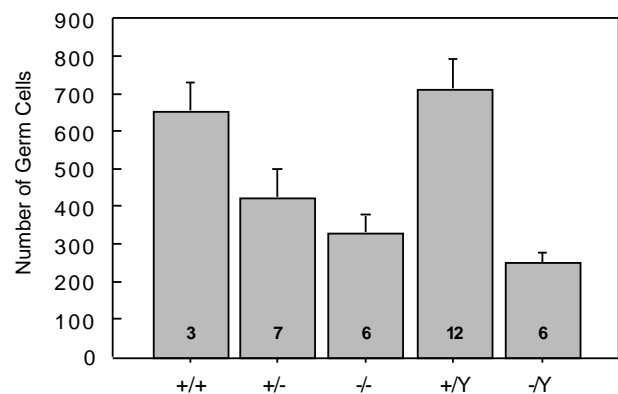

Fig. 5. Reduced primordial germ cell counts in *Zfx* mutant E11.5 embryos. Embryos of the five indicated genotypes were obtained from −/Y × +/− and +/Y × +/− matings. The mean number of primordial germ cells per embryo (± s.e.m.) is shown for each genotype; sample sizes are indicated on the bars.

lacking c-*jun* are of normal size (Hilberg et al., 1992; Johnson et al., 1993).

Embryonic growth retardation has been observed in mice lacking either insulin-like growth factor 1 (IGF-1), or IGF-2, or the type-1 IGF receptor (IGF1R), or insulin receptor substrate-1 (IRS-1). However, in these mutants, unlike *Zfx* mutant animals, the weight deficit (as a percentage of wild-type weight) becomes more marked during later embryonic development or postnatal life (DeChiara et al., 1990; Baker et al., 1993; Powell-Braxton et al., 1993; Araki et al., 1994; Tamemoto et al., 1994).

While *Zfx* appears to have a role in embryonic growth, we have found no convincing evidence that *Zfx* is involved in cell differentiation. Though *Zfx* mutant animals are small and have reduced numbers of germ cells, we detected no consistent qualitative pathological changes in any organ. In particular, as mentioned earlier, we detected no abnormalities of somatic sexual differentiation in males or females. The quantitative germ cell deficits in males and females could be explained by a role for *Zfx* in early embryonic growth, perhaps prior to germ cell allocation (as discussed below). As judged by skeletal maturation, development was temporally unaffected in *Zfx* mutant embryos. It is possible that the poorly understood, incomplete lethality observed in −/− and −/Y mice reflects a specific differentiation function, but we have no anatomical or histological evidence to support this conjecture. Thus, our study of mutant phenotypes is consistent with *Zfx* having little function in cell differentiation, strictly defined. Of course, if the present mutation does not result in loss of all *Zfx* function, then it remains possible that a complete loss-of-function allele would yield specific defects in cell differentiation.

Functional differences between mouse *Zfx* and *Zfy* genes

We have found that an intact Y chromosome, carrying wild-type copies of the *Zfy1* and *Zfy2* genes, fails to complement the growth deficit associated with the *Zfx* mutation. At E12.5 and E15.5, for example, the weights of −/Y and −/− embryos are indistinguishable from each other, though both are 20% below those of age-matched +/Y and +/− embryos (Table 2). We conclude that while *Zfx* plays a critical role in embryonic growth prior to E12.5, the *Zfy* genes do not provide the same growth function, at least in mice. Similarly, we find that the *Zfy* genes fail to complement the poor neonatal viability associated with the *Zfx* mutation. Both −/Y and −/− pups exhibited very poor viability, with only one tenth of either genotypic class surviving to weaning (Table 1, experiments 4 and 5). Perhaps the organismal functions of the murine *Zfy* genes are more restricted than those of *Zfx*. This might be understood in terms of previous reports concerning gene expression: while the murine *Zfx* gene is transcribed in a wide variety of embryonic, newborn, and adult tissues, the murine *Zfy* genes are transcribed in a much more restricted fashion, most prominently in testes (Koopman et al., 1989; Mardon and Page, 1989b; Mardon et al., 1990; Nagamine et al., 1990; Zambrowicz et al., 1994). Note that the *Zfx* mutant animals we have studied have two wild-type copies of *Zfa*, an expressed autosomal retroposon derived from *Zfx* (Page et al., 1987; Mardon et al., 1989a; Mitchell et al., 1989; Nagamine et al., 1989; Ashworth et al., 1990; Mardon et al., 1990; Page et al., 1990). The phenotypes of *Zfx* mutant, *Zfa* wild−type animals imply that the critical organismal functions of the *Zfx* and *Zfa* genes are not fully redundant.

Germ cell numbers and embryonic growth

Our observations indicate that *Zfx* plays a critical role in the growth or maintenance of germ cell populations in both males and females, and prior to gonadal sex differentiation. At E11.5, just before the onset of testicular or ovarian differentiation, PGC counts in both −/Y and −/− embryos were roughly half those observed in wild-type embryos. (The Y chromosome – and the *Zfy* genes – evidently fail to complement this PGC deficit, just as they fail to complement the growth and viability deficits associated with the *Zfx* mutation.) Spermatogenesis was qualitatively normal in −/Y adults, which were fertile, but spermatogenic output was halved. Oogenesis, maturation and ovulation appeared to be qualitatively normal in many young −/− females, but oocyte numbers and fertility were markedly diminished. In wild−type XX and XY embryos, PGCs derive from the epiblast/primitive ectoderm, are first recognizable in the extraembryonic mesoderm at about e7-8, and proliferate during their migration to the primitive gonad, which is largely complete by E11. Our observations suggest that the *Zfx* mutation perturbs one or more of the processes (allocation, migration, and proliferation) that establish and expand the germ cell pool during this period in embryogenesis.

If wild-type *Zfx* function is required for normal growth of the epiblast, then the low PGC counts in E11.5 embryos could be explained by a simple reduction in the number of epiblast cells allocated to the germ cell lineage. This early embryonic growth hypothesis could economically account for both the reduced body size and the germ cell deficits observed in *Zfx* mutant animals. (In utero exposure of early mouse embryos to mitomycin C, a DNA-alkylating agent that inhibits cell division, results in embryonic growth retardation and a striking decline in germ cell numbers in both sexes – with no specific differentiation defects – all reminiscent of the *Zfx* mutant phenotype; Tam and Snow, 1981) In −/− females, the oocyte deficits at 4 to 5 days post partum appeared to be much more severe than the PGC deficits at E11.5, suggesting that the *Zfx* mutation may also have impaired germ cell proliferation or survival during the period between these two timepoints.

A potential model for human ovarian failure

The *Zfx* mutant mouse may prove to be a useful model system for human ovarian failure since both share several characteristics: diminished numbers of oocytes, shortened reproductive lifespan, and an association with the X chromosome. Ovarian failure is a principal component of Turner syndrome and also an isolated finding in many infertile but otherwise healthy women. Ovarian histology in Turner syndrome and in isolated ovarian failure is reminiscent of that seen in *Zfx* mutant female mice, with oocyte numbers falling to negligible levels prematurely – this can happen before the age of puberty, in which case menses never begin, or later, resulting in premature menopause (Lippe et al., 1993). Moreover, the human homolog *ZFX* is on the X chromosome, a frequent site of microscopically detectable anomalies not only in Turner syndrome (classically associated with an XO karyotype) but also in otherwise healthy women with ovarian failure (Krauss et al., 1987; Tharapel et al., 1993). In humans, there is little opportunity to explore the embryologic origins of the oocyte deficit. The *Zfx*

mutant mouse provides an embryologically manipulable, molecularly defined context in which to explore fundamental and clinically relevant issues in germ cell development.

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