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Expression of Zinc Finger Protein 105 in the Testis and its Role in Male Fertility

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Abstract

Using an *in silico* approach, we identified a putative zinc finger domain-containing transcription factor (zinc finger protein 105, ZFP105) that was enriched in the adult mouse testis. RT-PCR analyses showed that *Zfp105* was indeed highly expressed in adult mouse testis and that its expression was regulated during postnatal development. To further characterize *Zfp105* expression, we generated a *Zfp105:*β*-galactosidase* (LacZ) knock-in reporter mouse line ($Zfp105^{LacZ/+}$) in which a *Zfp105:LacZ* fusion gene was expressed. Whole-mount LacZ analyses of adult $Zfp105^{LacZ/+}$ tissues showed robust LacZ staining in the testis, very weak staining in the ovary and no staining in the spleen, liver, kidney, heart, lung, thymus, adrenal gland, uterus or oviduct. Sectional LacZ staining showed that ZFP105 was highly expressed in pachytene spermatocytes. ZNF35, the human ortholog of ZFP105, was also expressed in male germ cells of normal human testis. More importantly, reduced male fertility and sloughed spermatogenic cells were observed in adult $Zfp105^{LacZ/LacZ}$ mice. Taken together, our results suggest that ZFP105 is a male germ-cell factor and plays a role in male reproduction.

Keywords

knock-in; LacZ; ZNF35; spermatocyte; mouse

Introduction

Spermatogenesis is the process that occurs in the seminiferous tubules of the testis leading to the production of mature sperm (Setchell 1993). This process includes the spermatogonial phase, which involves the proliferation of cells via mitosis, the spermatocyte phase, in which recombination and meiosis occurs to yield haploid cells, and the spermatid stage when terminal differentiation of the haploid spermatids yields mature sperm (Setchell 1993). This complex process is at least partially controlled by transcriptional factors in the spermatocytes and round spermatids (Kimmins et al. 2004; Li et al. 2005; Maclean and Wilkinson 2005). Therefore, characterization of transcriptional factors in these male germ cells will provide insights into normal spermatogenesis and ultimately the pathogenesis of certain types of male infertility.

As part of our goal to characterize the transcriptional regulation network during spermatogenesis and male reproduction, we used an *in silico*-based approach (Lin and

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Matzuk 2005; Zhang et al. 2008) to identify candidate transcriptional factors. Using this strategy, we identified a testis-enriched putative transcriptional factor, zinc finger protein 105 (ZFP105). ZFP105 is a 58 kilodalton protein that contains 11 C_2H_2-type zinc finger domains located at the carboxyl (C)-terminus. These zinc finger domains are conserved among their orthologs in other vertebrates (www.ensembl.org). The human ortholog, ZNF35, can bind to the DNA sequence 5'-C/GC/GAAG/TA-3' and likely functions as a transcriptional activator (Lanfrancone et al. 1992; Pengue et al. 1993). It has been proposed that ZFP105 or ZNF35 will play a critical role in myeloid terminal differentiation, early embryonic development, spermatogenesis and/or tumorigenesis (Kohno et al. 1993; Lanfrancone et al. 1992; Okada et al. 2005; Pannuti et al. 1988; Przyborski et al. 1998; Yoshikawa et al. 2006). In this report, we analyzed *Zfp105* mRNA expression in mouse tissues during postnatal development. More importantly we generated *Zfp105:galactosidase* (LacZ) reporter mice (*Zfp105*$^{LacZ/+}$) and characterized the expression pattern of ZFP105 in adults. Expression of *ZNF35*, the human ortholog of *Zfp105*, in the testis was also characterized. Finally, fertility and histological analyses were performed on homozygous *Zfp105:LacZ* mice. Our results suggest that ZFP105 is a male germ cell factor and plays a role in normal male fertility.

Results

Dominant expression of Zfp105 in the mouse testis

To identify genes enriched in the mouse testis, we performed the cDNA Digital Gene Expression Displayer (DGED) analysis of mouse cDNA libraries (http://cgap.nci.nih.gov/Tissues/GXS), similar to the approach described previously (Lin and Matzuk 2005; Zhang et al. 2008). Expression of these genes was then validated using mouse microarray data generated from the Genomics Institute of the Novartis Research Foundation (http://symaltas.gnf.org) (Su et al. 2002). Using this strategy, we identified a testis-enriched gene, *Zfp105*. Multi-tissue mouse microarray data (http://symatlas.gnf.org/SymAtlas) show that *Zfp105* is predominantly expressed in the testis (Su et al. 2002) (Figure 1A). To confirm the dominant expression of *Zfp105* in the testis, we performed RT-PCR analyses with specific primers for *Zfp105* and found the presence of abundant PCR products in adult mouse testes (Figure 1B). Weak expression of *Zfp105* was also detected in the ovary (Figure 1B). However, there was no detectable *Zfp105* expression in all other tested tissues (Figure 1B). These results are consistent with the above *in silico* prediction as well as a previous Northern blot analysis reported by Przyborski *et al* (Przyborski et al. 1998), indicating that *Zfp105* is primarily expressed in the mouse testis at the mRNA level.

Zfp105 expression in the mouse testis during postnatal development

To investigate *Zfp105* expression during postnatal development, we performed quantitative RT-PCR (QRT-PCR) analyses of RNA isolated from mouse testes at day 5, 9, 12, 14, 21, 28 and 56 after birth. As shown in Figure 1C, low levels of *Zfp105* mRNA were detected in testes of 5–12-day-old mice. However, high levels of *Zfp105* mRNA were detected in testes of 14–56-day-old mice (Figure 1C). These results indicate that *Zfp105* expression is developmentally regulated in the testis. Because pachytene spermatocytes are developed in 14-day-old or older testes but not in 12-day-old testes (Bellve 1993), our QRT-PCR studies (Figure 1C) indicate that *Zfp105* is likely expressed in pachytene spermatocytes at the mRNA level.

Generation of Zfp105$^{LacZ/+}$ reporter mice

To characterize *Zfp105* expression at the protein level, we performed immunohistochemical studies with commercial antibodies against the human ortholog ZNF35 and our own antibodies generated against bacterially-expressed recombinant ZFP105. However, neither

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antibody detected specific signals in mouse tissues (data not shown). To circumvent the lack of specific ZFP105 antibodies, we generated a *Zfp105:LacZ* reporter mouse line using the Baygenomic gene-trap embryonic stem (ES) cell line TEA059. Gene trapping is a method of generating ES cells with insertional mutations (Stanford et al. 2001). Baygenomic gene trap ES cells are generated by insertion of a gene-trap vector which contains a splice-acceptor sequence upstream of a reporter gene, *LacZ/Neomycin (Neo)* (a fusion of *LacZ* and *Neo*) into introns, resulting in the creation of a fusion transcript containing sequence from exon(s) upstream to the insertion joined to the *LacZ/Neo* marker (Stryke et al. 2003). To validate the correct insertion of the gene-trap *LacZ/Neo* vector into the *Zfp105* allele in the Baygenomic ES cell line TEA059, we performed genomic PCR analyses using primers P1 (recognizing DNA sequence upstream pf the insertion site of the gene-trap vector in *Zfp105* allele) and P3 (recognizing the gene-trap vector) (Figure 2A). As shown in Figure 2B, a specific 500-bp PCR product was detected in $Zfp105^{LacZ/+}$ but not wild type ES cells. These results demonstrate correct insertion of the reporter into the *Zfp105* allele in the TEA059 ES cell line. To investigate whether the *LacZ* reporter can be expressed in the TEA059 ES cell line, X-gal assays were performed on cultured ES cells with or without treatment of all-*trans*-retinoic acid (RA). As shown in Figure 2C, there was weak LacZ staining in untreated $Zfp105^{LacZ/+}$ ES cells. However, robust blue signals were observed in RA-treated $Zfp105^{LacZ/+}$ ES cells. These results suggest that *LacZ* reporter gene can be expressed in differentiated TEA059 cells. To test whether *LacZ* reporter gene expression in RA-treated TEA059 cells reflects endogenous *Zfp105* expression, we performed RT-PCR analyses to examine *Zfp105* expression in wild type ES cells after RA treatment. As shown in Figure 2D, increased *Zfp105* RNA levels were observed in RA-treated ES cells. $Zfp105^{LacZ/+}$ ES cells (TEA059) were then injected into mouse blastocysts to generate chimeric mice. Breeding of chimeric males with wild type C57BL/6 females produced agouti pups. Genotype analyses of mouse tails from the agouti pups showed successful germline transmission of the $Zfp105^{LacZ}$ allele (Figure 2E). To determine whether the expression of *LacZ* is driven by the endogenous *Zfp105* promoter in $Zfp105^{LacZ/+}$ mice, we performed RT-PCR analysis of $Zfp105^{LacZ/+}$ testes using a forward primer recognizing the exon 2 of *Zfp105* and a reverse primer recognizing the *LacZ* gene (Figure 2F). As shown in Figure 2G, a 1.8-kb band was detected in $Zfp105^{LacZ/+}$, but not in $Zfp105^{+/+}$ testes. These results suggest that *Zfp105:LacZ* fusion gene is expressed in $Zfp105^{LacZ/+}$ mice. Collectively, a *Zfp105:LacZ* reporter mouse line has been successfully generated.

ZFP105 expression in adult $Zfp105^{LacZ/+}$ mice

Having generated $Zfp105^{LacZ/+}$ reporter mice, we performed whole-mount LacZ staining on various tissues from adult $Zfp105^{+/+}$ and $Zfp105^{LacZ/+}$ mice to determine ZFP105 expression (Figure 3A). As expected, there was no LacZ staining in tissues from control $Zfp105^{+/+}$ mice (data not shown). In $Zfp105^{LacZ/+}$ mice, strong positive staining was observed in the testis, but not in heart, kidney, liver, lung, spleen, thymus, adrenal gland, oviduct or uterus (Figure 3A). Weak LacZ staining was also observed in a few follicles of $Zfp105^{LacZ/+}$ ovaries (Figure 3A). To further determine ZFP105 expression in the testis, we performed LacZ staining on frozen sections from $Zfp105^{+/+}$ and $Zfp105^{LacZ/+}$ mice (Figure 3B). As expected, there was no positive LacZ staining in $Zfp105^{+/+}$ testis. In $Zfp105^{LacZ/+}$ mice, there was very weak staining in interstitial cells, spermatogonia and round and elongating spermatids within the seminiferous tubules (Figure 3B). However, robust staining was observed in the spermatocytes (*esp*. pachytene spermatocytes) of adult $Zfp105^{LacZ/+}$ testes (Figure 3B). These results suggest that ZFP105 is highly expressed in the testis with dominant expression in the spermatocytes.

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Expression of ZNF35, the Zfp105 human ortholog, in normal human testis

To investigate whether *ZNF35* has an expression pattern similar to its mouse ortholog *Zfp105*, we first performed RT-PCR analyses using specific primers for *ZNF35*. As shown in Figure 4A, high levels of *ZNF35* transcripts were detected in normal human testis, but not in other tested tissues including spleen, thymus, prostate, ovary, small intestine, colon and leukocytes. Then, immunofluorescence was performed using specific ZNF35 antibodies to examine ZNF35 expression in human testes. As shown in Figure 4B, no positive fluorescence signals were detected in the testis with normal rabbit IgG. However, positive fluorescence signals with anti-ZNF35 antibodies were detected in male germ cells within seminiferous tubules as well as in interstitial cells. Positive nuclear staining was detected in only a limited number of male germ cells (indicated by the arrow in Figure 4B), while the majority of male germ cells displayed cytosolic or perinuclear staining. The above results indicate that like *Zfp105*, *ZNF35* is also highly expressed in the testis, particularly in the spermatogenic cells.

Reduced male fertility in Zfp105$^{LacZ/LacZ}$ mice

To test whether ZFP105 plays a role in male fertility, *Zfp105*$^{LacZ/LacZ}$ mice were generated and then subjected to fertility analysis. As shown in Figure 5A, homozygous LacZ reporter mice were successfully generated from the intercross of *Zfp105*$^{LacZ/+}$ mice. There was no overt developmental defect in *Zfp105*$^{LacZ/LacZ}$ mice. Northern blot analysis using radiolabeled probes against exon 4 (encoding 11 C_2H_2 domains) showed the complete loss of RNA transcripts in *Zfp105*$^{LacZ/LacZ}$ testes (Figure 5B), indicating that the putative DNA binding domains of ZFP105 are absent in *Zfp105*$^{LacZ/LacZ}$ mice. To test if there is a fertility defect in *Zfp105*$^{LacZ/LacZ}$ mice, we performed fertility analysis of mutant mice and their wild type and heterozygous littermates for six-eight months. As shown in Figure 5C, no significant change in the number of litters per month was observed among tested *Zfp105*$^{+/+}$, *Zfp105*$^{LacZ/+}$, *Zfp105*$^{LacZ/LacZ}$ males or females during the breeding period. However, there was a significant decrease in the litter size produced from male *Zfp105*$^{LacZ/LacZ}$ mice, when compared to wild type and heterozygous males and homozygous female littermates ($P < 0.05$). These results suggest that loss of exon 4 from *Zfp405* causes male subfertility.

Sloughed spermatogenic cells in Zfp105$^{LacZ/LacZ}$ testes

To investigate whether there is a morphological defect in the testis, we performed histological analyses of testes and epididymides from *Zfp105*$^{LacZ/LacZ}$ and control *Zfp105*$^{LacZ/+}$ littermates. All stages of seminiferous tubules during spermatogenesis were observed in *Zfp105*$^{LacZ/LacZ}$ and control *Zfp105*$^{LacZ/+}$ littermates (data not shown). However, sloughed spermatogenic cells were present in the lumen of seminiferous tubules in *Zfp105*$^{LacZ/LacZ}$ testes but not in control littermates (Figure 5D, top two panels). Sloughed cells (likely the spermatogenic cells) in the epididymal lumen were also observed in *Zfp105*$^{LacZ/LacZ}$ but not *Zfp105*$^{LacZ/+}$ littermates (Figure 5D, bottom two panels). These results suggest that *Zfp405* plays a role in normal spermatogenesis.

Discussion

Using an *in silico* approach (Lin and Matzuk 2005; Zhang *et al*. 2008), we have identified a putative transcription factor ZFP105 enriched in the testis. Consistent with the *in silico* prediction and previous studies (Przyborski et al. 1998), *Zfp105* is highly expressed in the testis (Figure 1B). Our QRT-PCR studies of *Zfp105* mRNA in the testes during postnatal development (Figure 1C) suggest that *Zfp105* is highly expressed in pachytene spermatocytes, given that pachytene spermatocyte is the latest stage of spermatogenic cells developed in the testis at 14 days after birth (Bellve 1993). Indeed, *Zfp105* transcripts have been detected in pachytene spermatocytes by *in situ* hybridization and Northern blot

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analyses (Przyborski et al. 1998). More importantly, we have successfully generated *Zfp105:LacZ* reporter mice in which the *Zfp105:LacZ* fusion gene is expressed (Figure 2G). LacZ expression in adult *Zfp105*$^{LacZ/+}$ tissues (Figure 3) and RA-treated gene-trap *Zfp105* ES cells (Figure 2C) are consistent with *Zfp105* mRNA expression in adult mouse tissues (Figure 1B) (Przyborski *et al*. 1998) and wild type ES cells (Figure 2D), respectively. Induced *Zfp105* expression in RA-treated ES cells (Figure 2C–D) is also consistent with the original identification of *Zfp105* (Przyborski *et al*. 1998) using a probe against a zinc finger transcript upregulated in RA-treated human embryonal carcinoma NTERA2 cells (Andrews 1984). Robust LacZ expression in pachytene spermatocytes of adult *Zfp105:LacZ* reporter mice correlates well with *Zfp105* mRNA expression in those cells (Przyborski *et al*. 1998), even though our LacZ expression results in postmeoitic spermatids of *Zfp105*$^{LacZ/+}$ testes are different from *Zfp105* mRNA expression as reported previously (Przyborski *et al*. 1998). Together, our results suggest that *Zfp105* is highly expressed in the testis, especially in pachytene spermatocytes. Like *Zfp105*, *ZNF35* was also highly expressed in human testis and ZNF35 protein was detected in spermatogenic cells (Figure 4A–B). Collectively, we have successfully characterized the germ cell-expression of *Zfp105* and its human ortholog in adult testes.

Male germ-cell expression of *Zfp105* and *ZNF35* characterized here suggests that they may play a critical role in spermatogenesis and affect male fertility. Indeed, we have observed reduced fertility in homozygous *Zfp105:LacZ* reporter male mice (Figure 5C). Reduced male fertility is likely caused by sloughing of immature spermatogenic cells into the testicular lumen in *Zfp105*$^{LacZ/LacZ}$ mice (Figure 5D). Our results suggest that ZFP105 plays a role in normal spermatogenesis and ultimately male fertility. However, the underlying molecular mechanism of ZFP105 action during spermatogenesis and male fertility remains to be elucidated. One potential mechanism of ZFP105 action in spermatogenic cells, however, is to bind to the putative ZFP105/ZNF35 binding sites (5'-C/GC/GAAG/TA-3') (Pengue et al. 1993) and control the transcription of male germ-cell-specific genes. Since *Zfp105* expression can be induced by RA in ES cells (Figure 2C–D), it is possible that a similar scenario occurs in the adult testis. ZFP105 could be a target of RA signaling that are important for normal spermatogenesis (Chung *et al*. 2004; Dufour and Kim 1999; Kastner *et al*. 1996). In addition, ZFP105 may function as an RNA binding protein like other zinc finger proteins such as REX-1/ZFP42 (Rogers *et al*. 1991), KIN 17 (Pinon-Lataillade et al. 2004) and ZFR (Meagher *et al*. 1999) to elicit its biological function in spermatogenic cells (*e.g.* spermatocytes). Regardless, our studies on *Zfp105*$^{LacZ/LacZ}$ mice provide direct evidence about the role of ZFP105 in male fertility.

It should be emphasized here that *Zfp105*$^{LacZ/LacZ}$ mice unlikely are *Zfp105* null mutants. This is indicated by the presence of *Zfp105:LacZ* fusion transcripts in *Zfp105*$^{LacZ/+}$ testes (Figure 2F–G). Although full-length *Zfp105* RNA transcripts were not detected in *Zfp105*$^{LacZ/LacZ}$ testes by radiolabeled probes against the C_2H_2 domain-coding cDNA (exon 4) (Figure 5B), the first 110 amino-acid residues of ZFP105 (encoded by the exons 2 and 3 of *Zfp105*) could be translated from the *Zfp105:LacZ* fusion transcripts and have a role in male fertility. This may explain why there is a relatively minor reduction in male fertility displayed in *Zfp105*$^{LacZ/LacZ}$ mice. In addition, there exists a ZNF35 paralog, ZNF660 that contains 10 C_2H_2 domains, in human genome (www.ensembl.org). The potential existence of the *Zfp105* paralog, *Zfp660*, could compensate the loss of the C-terminus of ZFP105 in *Zfp105*$^{LacZ/LacZ}$ mice and mask the male fertility phenotypes. Furthermore, many other C_2H_2-type zinc finger proteins such as ZFR (containing 3 C_2H_2 repeats), ZFP42 (containing 4 $C2H_2$ repeats) and KIN17 (containing 11 $C2H_2$ domain) have been shown to be expressed in mouse spermatocytes (Meagher *et al*. 1999; Pinon-Lataillade *et al*. 2004; Rogers *et al*. 1991). These zinc finger proteins may have some functional redundancies with ZFP105 in spermatocytes to regulate spermatogenesis and male fertility. Nevertheless, our studies

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suggest that the C-terminus of ZFP105 (containing 11 C_2H_2 domains encoded by single exon 4) plays a role in normal spermatogenesis and male fertility.

It has been postulated that *Zfp105* may be involved in early embryonic development and tumorigenesis (Kohno *et al*. 1993; Lanfrancone *et al*. 1992; Okada *et al*. 2005; Pannuti *et al*. 1988; Przyborski *et al*. 1998; Yoshikawa *et al*. 2006). However, embryonic lethality was not observed in *Zfp105*$^{LacZ/LacZ}$ mice. There were no gross defects in *Zfp105*$^{LacZ/LacZ}$ mice during postnatal development. We also did not observe any tumor in the testis or other major organs from *Zfp105*$^{LacZ/LacZ}$ mice up to 12 months of age (data not shown). Therefore, our studies indicate that the C-terminal ZFP105 encoded by the exon 4 is not important for normal embryonic development and tumorigenesis, except the aforementioned male fertility.

In conclusion, we have successfully identified and characterized *Zfp105* expression in adult mice. Our results suggest that ZFP105 is a male-germ cell factor in adults. More importantly, our studies provide direct evidence on the role of *Zfp105* in male fertility.

Materials and methods

In silico analysis

Mouse cDNA sequences enriched in the testis were obtained using the cDNA Digital Gene Expression Displayer (DGED) program (http://cgap.nci.nih.gov/Tissues/GXS), similar to previous reports (Lin and Matzuk 2005; Zhang *et al*. 2008) . Expression of these genes in the testis was then examined on multi-tissue mouse microarray data generated from the Genomics Institute of the Novartis Research Foundation (http://symaltas.gnf.org) (Su *et al*. 2002).

ES cell culture

Gene-trap *Zfp105* ES cells (TEA059) were purchased from BayGenomics (University of California at Davis, California). These cells were cultured in ES cell media containing 400 μg/ml G418 (Invitrogen, Carlsbad, CA) on irradiated SNL feeder cells expressing murine leukemia inhibitory factor, as described previously (Mudgett and Livelli 1995). Wide type (AB1.2) and gene-trap *Zfp105*$^{LacZ/+}$ ES cells were incubated with 10^{-6} M RA (Sigma Chemical Co., St. Louis, MO) to induce differentiation.

Animals, genotyping and tissue collection

Adult C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and maintained on a 14 hour light:10 hour dark cycle, with free access to food and water, in the vivarium of the University of Louisville. *Zfp105*$^{LacZ/+}$ ES cells were injected into C57BL/6 blastocysts to generate male chimeras with 50–100% agouti color. These chimeric mice were bred with female C57BL/6 mice to generate *Zfp105*$^{LacZ/+}$ mice. *Zfp105*$^{LacZ/+}$ mice were then intercrossed to obtain *Zfp105*$^{LacZ/LacZ}$ mice. Genomic DNA of *Zfp105*$^{LacZ/+}$ ES cells and mouse tails were extracted and genotyped by PCR (Lan et al. 2003c) using primers 5'-atgtgtttgcatgggtcaga-3' (P1), 5'-aaaggggaagggcaaaacta-3' (P2) and 5'-aaaggggaagggcaaaacta-3' (P3). Animals were euthanized with carbon dioxide and tissues were collected for RNA isolation, LacZ staining or fixation. Frozen sections were prepared as described previously (Zhang et al. 2008). The stage of seminiferous tubules within the testis was determined as described previously (Russell et al. 1990). All animal studies were conducted according to ethical guidelines and approved by the Animal Welfare and User Committee of University of Louisville.

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RNA isolation, RT-PCR and Northern blot analysis

Total RNA from mouse tissues and RA-treated ES cells was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). Semi-quantitative RT-PCR analyses with specific primers for *Zfp105, Zfp105:LacZ fusion gene* and β-*actin* were performed as described previously (Lan et al. 2003b). PCR analyses of *ZNF35*, and glyceraldedyde-3-phosphate dehydrogenase (GAPDH) were performed on normal human tissue cDNA (Catalog# K1421–1, Clontech Laboratories, Inc., *ZNF35*, and glyceraldedyde-3-phosphate dehydrogenase *(GAPDH)* Mountain View, CA). PCR primer sequences are as follows: *Zfp105*$^{+/+}$, 5'-taagtcaggcatccagccaac-3' (105-F) and 5'-ctcccaaatctccctttcctc-3' (105-R), 438-bp products; *Zfp105:LacZ* fusion gene, 5'-taagtcaggcatccagccaac-3' (105-F) and 5'-atacagcgcgtcgtgattag-3'(LacZ-R), 1.8-kb products; *ZNF35*, 5'-gcggtagtcctgaaagcaac-3' and 5'-caacgaggtttgcactctga-3', 499-bp products; *GAPDH*, 5'-tgaaggtcggagtcaacggatttggt-3' and 5'-catgtagggccatgaggtccaccac-3', 983-bp products; and β-*actin*, 5'-ttgagaccttcaacacccc-3' and 5'-agccagagcagtaatctcc-3', 593-bp products. QRT-PCR analyses were performed as described previously (Lan et al. 2003b). QRT-PCR primers and Taqman® probes for *Zfp105* are 5'-atggaagcatcagtgtatcagaagat-3', 5'-gcttttagcaccactgccatt-3', and 5'-FAM-aaagggtcaggaaatgttccggg-TAMRA-3' (Biosource International, Inc. Camarillo, CA). Northern blot analysis was performed using random-labeled probes against the exon 4 of *Zfp105* or β-*actin*, as described previously (Lan et al. 1999).

LacZ staining and immunofluorescent studies

LacZ staining of RA-induced ES cells or tissue samples was performed according to the manufacturers' protocol (Specialty Media, Phillipsburg, NJ). Immunofluorescent studies were performed on paraffin-embedded normal human testis sections (purchased from US Biomax, Inc, Rockville, MD) using anti-ZNF35 antibodies (GenWay Biotech, Inc., San Diego, CA), as described previously (Lan et al. 2003a).

Male fertility studies

Five to six *Zfp105*+/+, *Zfp105*$^{LacZ/+}$ and *Zfp105*$^{LacZ/LacZ}$ littermates were bred with proven fertile C57BL/6 females or males (1 male/1 female per cage) for 6–8 months. Litter sizes and litters per month were recorded as described previously (Lan et al. 2003b). *Student's* t-test was performed to determine the significant difference in litter size and the number of litters per month among each group.

Histological analysis

For histological analysis, tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin-embedded tissues were sectioned at 7-μm thickness using a Richard-Allan Scientific MICROM HM325 microtome (Fisher Scientific, Pittsburgh, PA), and then stained with hematoxylin and eosin (H & E). Stained sections were examined under an Axio-Imager A1 microscope (Carl Zeiss Inc, Gottingen, Germany). Sections from at least three animals of each genotype were analyzed.

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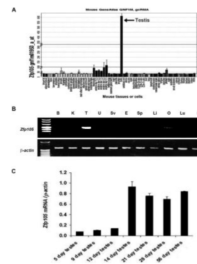

Figure 1.

Zfp105 expression in the mouse testis. **(A)** Microarray data from the Genomics Institute of the Novartis Research Foundation showing *Zfp105* expression in adult mouse testes (Su et al. 2002). **(B)** Expression of *Zfp105* in adult mouse testes by semi-quantitative RT-PCR analyses. Lanes: B, brain; K, kidney; T, testis; U, uterus; Sv, seminal vesicles; E, epididymis; Sp, spleen; Li, liver; O, ovary; Lu, lung; H, heart. PCR cycles: *Zfp105*, 26 cycles; β-*actin*, 22 cycles. **(C)** Testicular expression of *Zfp105* during postnatal development by QRT-PCR analyses. *Zfp105* mRNA levels were normalized by β-*actin* mRNA in each sample (mean ± standard errors, n=3).

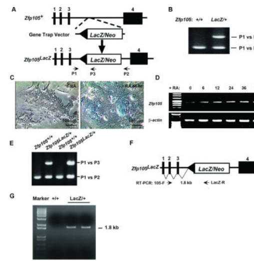

Figure 2.

Generation of *Zfp105*$^{LacZ/+}$ reporter mice. (**A**) Schematic representation of gene-trap *Zfp105*^{LacZ} allele. P1, P2 and P3 are the primers for genotyping. (**B**) Genomic PCR analyses (using primers P1 and P3) showing the correct insertion of the gene trap vector in the *Zfp105* allele. (**C**) Positive LacZ staining in *Zfp105*$^{LacZ/+}$ ES cells following RA treatment for 48 hours. (**D**) RT-PCR showing RA-induced *Zfp105* expression in wild type ES cells. The number of PCR cycles for *Zfp105* and β-*actin* were 26 and 23, respectively. (**E**) PCR analyses showing germline transmission of the *Zfp105*^{LacZ} allele. Tail DNA of four agouti pups produced from the breeding of a chimeric male with a C57BL/6 female were subjected to PCR analyses with the indicated PCR primers. (**F**) Schematic representation of the *Zfp105:LacZ* fusion transcript. RT-PCR primers are indicated by arrows. (**G**) RT-PCR analysis showing the expression of *Zfp105:LacZ* fusion RNA transcript in 2-month-old *Zfp105*$^{LacZ/+}$ testes.

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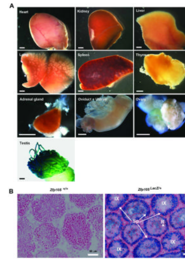

Figure 3.

LacZ staining of adult tissues from *Zfp105*$^{LacZ/+}$ mice. **(A)** Whole-mount LacZ staining of various tissues from adult *Zfp105*$^{LacZ/+}$mice. Note the weak staining in some ovarian follicles (indicated by the white arrow) and the robust staining in the testis. Scale bar = 1 mm. **(B)** LacZ staining in the spermatocytes of adult *Zfp105*$^{LacZ/+}$ (right panel) but not in *Zfp105*$^{+/+}$ testes (left panel). Sp, spermatocytes (arrows); R, round spermatids (arrowhead). In the right panel, tubules at stage IX are indicated by IX, while the other tubules are likely at stages V–VI.

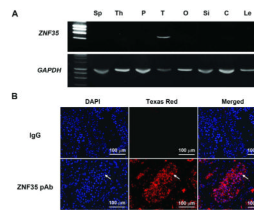

Figure 4.

ZNF35 expression in human testis. **(A)** RT-PCR showing high expression of *ZNF35* mRNA in normal human testis. Sp, spleen; Th, thymus; P, prostate; T, testis; O, ovary; Si, small intestine; C, colon; Le, leukocytes. PCR cycles: *ZNF35*, 31 cycles; *GAPDH*, 26 cycles. **(B)** Immunofluorescent studies showing the presence of ZNF35 in spermatogenic cells within human testis. Nuclear staining is indicated by the arrow.

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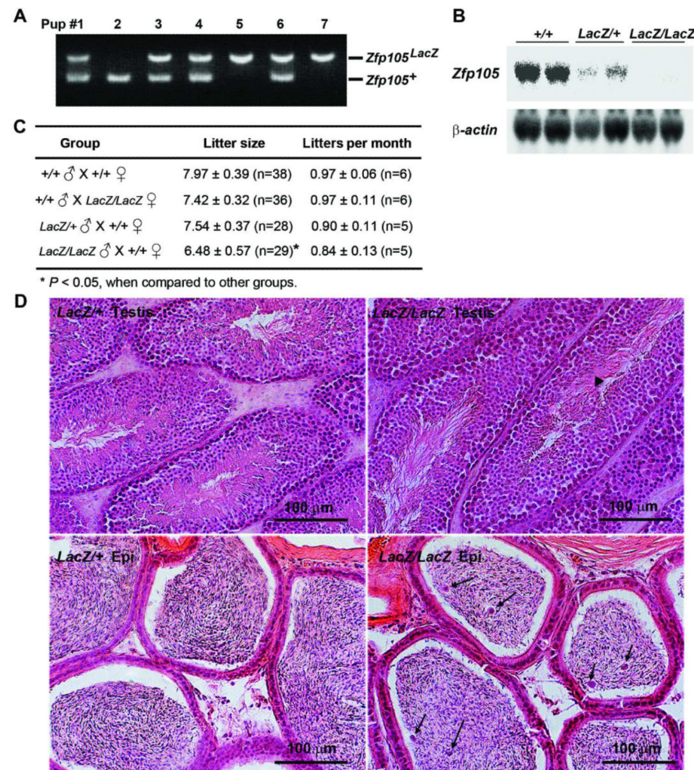

Group	Litter size	Litters per month
+/+ ♂ X +/+ ♀	7.97 ± 0.39 (n=38)	0.97 ± 0.06 (n=6)
+/+ ♂ X LacZ/LacZ ♀	7.42 ± 0.32 (n=36)	0.97 ± 0.11 (n=6)
LacZ/+ ♂ X +/+ ♀	7.54 ± 0.37 (n=28)	0.90 ± 0.11 (n=5)
LacZ/LacZ ♂ X +/+ ♀	6.48 ± 0.57 (n=29)*	0.84 ± 0.13 (n=5)

* $P < 0.05$, when compared to other groups.

Figure 5.

Fertility and histological analysis of *Zfp105*$^{LacZ/LacZ}$ mice. **(A)** Representative genotyping results of a litter of pups produced from heterozygous intercrosses. Pup #2, *Zfp105*$^{+/+}$; Pup# 1, 3, 4 and 6, *Zfp105*$^{LacZ/+}$; and Pup #5 and 7, *Zfp105*$^{LacZ/LacZ}$. **(B)** Northern blot analysis showing the loss of full-length *Zfp105* transcripts in *Zfp105*$^{LacZ/LacZ}$ testes using probes against the exon 4 of *Zfp105*. **(C)** Breeding results showing male subfertility in *Zfp105*$^{LacZ/LacZ}$ mice. Five to six mice of each genotype were bred with fertile males or females for a period of 6–8 months. Data are represented as mean ± standard error of the indicated number of samples. **(D)** Histological analysis of testes and epididymides of 3-month-old *Zfp105*$^{LacZ/LacZ}$ and control *Zfp105*$^{LacZ/+}$ littermates. Note the presence of sloughed spermatogenic cells in the lumen of seminiferous tubules (indicated by arrowhead) and epididymis (indicated by arrows) from *Zfp105*$^{LacZ/LacZ}$ mice.