

LETTER

Rfx2 Is Required for Spermatogenesis in the Mouse

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Summary: RFX transcription factors are key regulators of ciliogenesis in vertebrates. In *Xenopus* and zebrafish embryos, knockdown of *Rfx2* causes defects in neural tube closure and in left–right axis patterning. To determine the essential role of the *Rfx2* gene in mammalian development, we generated *Rfx2*-deficient mice using an embryonic stem cell clone containing a *lacZ* gene trap reporter inserted into the first intron of the *Rfx2* gene. We found that the *Rfx2 lacZ* reporter is expressed in ciliated tissues during mouse development including the node, the floor plate and the dorsal neural tube. However, mice homozygous for the *Rfx2* gene trap mutation did not have defects in neural tube closure or in organ *situs*. The gene trap insertion appears to create a null allele as *Rfx2* mRNA was not detected in *Rfx2^{gt/gt}* embryos. Although *Rfx2*-deficient mice do not have an obvious embryonic phenotype, we found that *Rfx2^{gt/gt}* males are infertile because of a defect in spermatid maturation at or before the round and elongating spermatid stage. Our results indicate that *Rfx2* is not essential for embryonic development in the mouse but is required for spermatogenesis. *genesis* 53:604–611, 2015. © 2015 Wiley Periodicals, Inc.

Key words: gene trap; ciliogenesis; node; neural tube; male fertility

RESULTS AND DISCUSSION

Cilia are microtubule-based organelles that project from the surface of cells and play an important role in cellular signaling (Berbari *et al.*, 2009). During development, cilia located in the node and in the floor plate are important in left–right axis formation and in dorsal–ventral patterning of the neural tube, respectively (Eggenchwiler and Anderson, 2007; Hirokawa *et al.*,

2006). Recent work indicates that the *Rfx2* transcription factor regulates the expression of a ciliogenic gene program necessary for proper neural tube closure in *Xenopus*, and for left–right axis specification in both zebrafish and *Xenopus* embryos (Bisgrove *et al.*, 2012; Chung *et al.*, 2012). To determine the essential role of the mammalian *Rfx2* gene we generated an *Rfx2* gene trap mouse line using a C57BL/6N gene-trap clone, IST10638H11, obtained from the Texas A&M Institute for Genomic Medicine. The ES cell clone contains a *lacZ* reporter poly A gene cassette with a splice acceptor inserted into intron 1 of the *Rfx2* gene before the ATG start codon in exon 2 (Fig. 1). Transcription at the locus should result in the splicing of exon 1 to the gene trap vector and eliminate the production of the endogenous transcript. This mutation is predicted to create a null allele and the expression of the *lacZ* gene should mimic the expression of the *Rfx2* gene.

To determine the expression pattern of *Rfx2*, we performed X-gal staining on embryos from timed matings between *Rfx2^{gt/+}* males and CD-1 females. At embryonic day (E) 7.0, *lacZ* activity was detected in the anterior primitive streak preceding the morphological appearance of the node, and at E7.5 in the node and in the midline notochordal plate cells extending anteriorly from the node (Fig. 2a,b). At E8.5, *lacZ* activity was present in the node now located in the tail region

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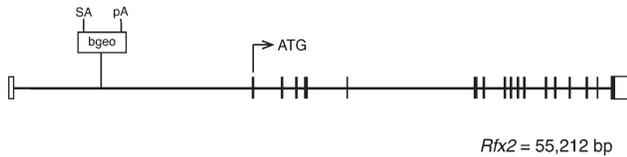


FIG. 1. *Rfx2* gene trap allele. The gene trap vector containing a β geo cassette with a splice acceptor (SA) and poly A (pA) sequence is inserted into intron 1 before the ATG start codon in exon 2. The β geo gene trap cassette is not drawn to scale.

(Fig. 2c), consistent with previous results using RNA *in situ* hybridization to *Rfx2* (Bisgrove *et al.*, 2012). That previous study of *Rfx2* expression in the mouse focused largely on expression in the node. Notably, using our *lacZ* allele, we also observed that at E9.5, X-gal staining was detected in the floor plate and in the dorsal portion of the neural tube, with the area of highest expression in the anterior portion of the spinal cord. Staining was also detected in the developing gut (Fig. 2d-f). At E10.5, an additional staining region was observed in the

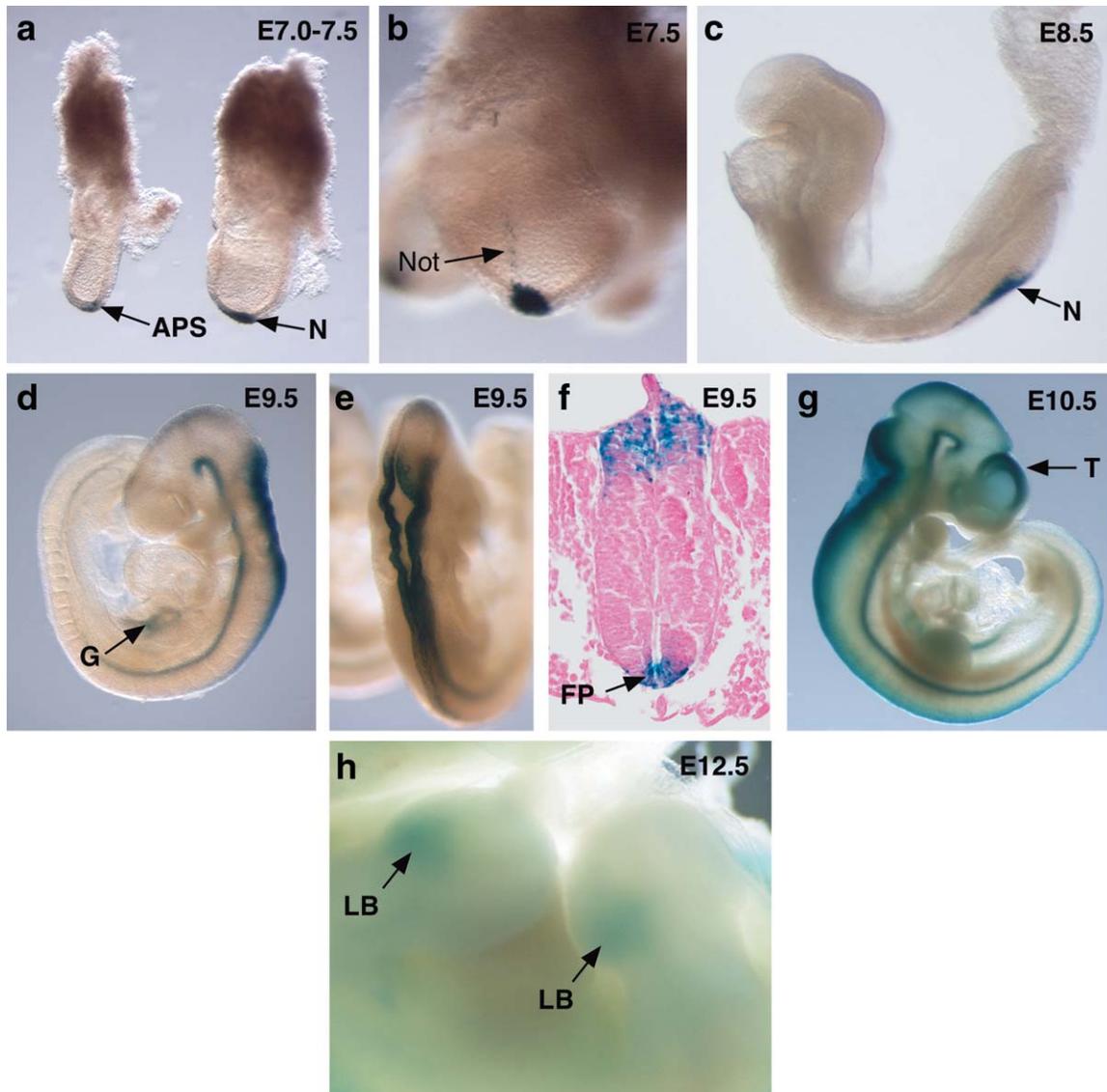


FIG. 2. Expression of *Rfx2* between E7.0 and E12.5 as revealed by X-gal staining for *lacZ* activity. **(a)** At E7.0–7.5 *lacZ* activity is present in the anterior primitive streak region prior to the morphological appearance of the node (E7.0) and then in the definitive node (E7.5). **(b)** E7.5 embryo showing *lacZ* activity in the node and the midline notochordal plate extending anteriorly from the node. **(c)** E8.5 embryo with *lacZ* activity in the node localized to the tail region. **(d–f)** E9.5 embryo showing *lacZ* activity in the dorsal neural tube and the floor plate and in the gut region. **(g)** E10.5 embryo with a new expression domain in the telencephalon. **(h)** E12.5 embryo with expression in the anterior portion of the fore and hind limb buds. APS = anterior primitive streak, N = node, Not = notochordal plate, G = gut, FP = floor plate, T = telencephalon, LB = limb bud.

Table 1
Genotype of Mice at Weaning from $Rfx2^{gt/+}$ x $Rfx2^{gt/+}$ Crosses

+/+	+/-	-/-
24 (27%)	43 (48%)	23 (26%)

Includes mice on both C57BL/6N and C57BL/6N x CD-1 backgrounds

telencephalon region of the brain and at E12.5 *lacZ* activity was also observed in the anterior portion of the limb bud (Fig. 2g,h).

To determine whether $Rfx2^{gt/gt}$ mice were viable we performed heterozygous intercrosses on both C57BL/6N and C57BL/6N x CD-1 genetic backgrounds and genotyped the resulting mice at weaning. We obtained wild-type, heterozygous and homozygous $Rfx2$ mice at approximately the expected ratio of 1:2:1 (Table 1). We also observed that the stomach, as indicated by the milk spot, was correctly located on the left side in all pups examined (76 pups, 11 litters) from heterozygous by heterozygous crosses. This suggests that the $Rfx2$ mutation does not cause *situs inversus*. We also examined embryos from matings between mice heterozygous for the gene trap allele at E10.5 and found no obvious morphological differences between $Rfx2^{+/+}$ and $Rfx2^{gt/gt}$ littermate embryos (data not shown). Adult $Rfx2^{gt/gt}$ mice appeared morphologically normal and did not display any obvious abnormal behaviors. These results indicate that mice homozygous for the $Rfx2$ gene trap allele are viable and do not have any obvious structural malformations.

One possible explanation for the lack of an embryonic phenotype is that the gene-trap mutation does not create a null allele. To determine whether $Rfx2$ mRNA is present in $Rfx2^{gt/gt}$ embryos we performed reverse transcription polymerase chain reaction (RT-PCR) using total RNA from E9.5 stage embryos. We used primers that amplify cDNA sequences downstream of the gene trap that correspond to exons 8 through 12, which encompasses part of the DNA binding domain of $Rfx2$. A band of the predicted size of 494 bp was observed in the $Rfx2^{+/+}$ cDNA sample but no band was observed in the $Rfx2^{gt/gt}$ cDNA sample (Fig. 3). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as a control for the quality of the cDNA samples and amplification was observed in both $Rfx2^{+/+}$ and $Rfx2^{gt/gt}$ samples. The absence of detectable $Rfx2$ mRNA suggests that the gene trap insertion creates a null allele and that $Rfx2$ is not essential for embryonic development in the mouse. We also note that embryonic development appears to be normal in an independently generated $Rfx2$ knockout mouse line (Kistler *et al.*, 2015).

To determine whether mice homozygous for the gene trap were fertile, we crossed four $Rfx2^{gt/gt}$ females with littermate $Rfx2^{+/+}$ males and eight $Rfx2^{gt/gt}$ males with $Rfx2^{+/+}$ females. All four $Rfx2^{gt/gt}$ females were

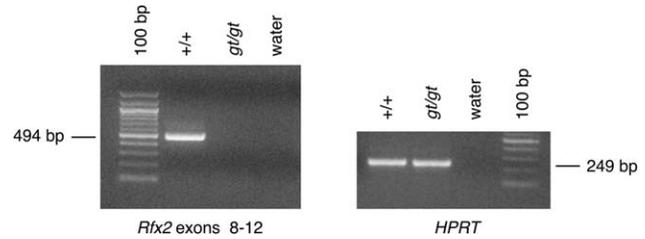


FIG. 3. RT-PCR analysis of $Rfx2$ expression in E9.5 $Rfx2^{+/+}$ and $Rfx2^{gt/gt}$ embryos. PCR was performed with primers that amplify sequences spanning exons 8 through 12. The amplified $Rfx2$ cDNA band is 494 base pairs (bp). In the mouse genome the primer sequences are separated by 2993 bp of genomic DNA. *HPRT* was used as a positive control for the quality of the synthesized first strand cDNA and amplify a band of 249 bp. The *HPRT* primers amplify a band of ~1100 bp in genomic DNA (not shown).

fertile, however none of the eight $Rfx2^{gt/gt}$ males were fertile although all displayed normal mating and plugging behavior. $Rfx2^{gt/gt}$ males on both C57BL/6N and C57BL/6N x CD-1 genetic backgrounds were analyzed. We next attempted to recover sperm from the caudal epididymis of 12 week old $Rfx2^{gt/+}$ and $Rfx2^{gt/gt}$ males. Although abundant sperm was recovered from littermate $Rfx2^{gt/+}$ males, $Rfx2^{gt/gt}$ males completely lacked sperm. To determine whether the lack of sperm in the epididymis was because of a defect in sperm production or sperm transport to the epididymis, we examined histological sections of testes from $Rfx2^{gt/+}$ and $Rfx2^{gt/gt}$ mice. We observed abundant spermatozoa in the seminiferous tubules of $Rfx2^{gt/+}$ mice but no spermatozoa was present in the seminiferous tubules of $Rfx2^{gt/gt}$ mice. Instead, we observed an accumulation of abnormal symplasts of round spermatids (Fig. 4a-d). Spermatogonial cells and spermatocytes were present in the $Rfx2^{gt/gt}$ testis and were not obviously abnormal. We did note that in the $Rfx2^{gt/gt}$ testis sections there appeared to be an increase in the number of Leydig cells in the interstitial space (Fig. 4e,f).

To confirm that early spermatid development was affected, we analyzed the expression of several marker genes expressed in spermatocytes and spermatids by quantitative PCR (qPCR). The transcription factor genes *A-myb* (*Mybl1*) and *Crem* encode transcription factors expressed in spermatocytes (Foulkes *et al.*, 1992; Mettus *et al.*, 1994). Both *A-myb* and *Crem* (*Crem-tau* isoform) were expressed in the testis of $Rfx2^{gt/gt}$ mice at levels roughly equivalent to levels in the testis from $Rfx2^{+/+}$ mice (Fig. 5a,b). We next examined the expression of *Tnp1* and *Tnp2*, which encode proteins involved in chromatin condensation and whose mRNAs are present in round and elongating spermatids (Kleene, 1989). We found that *Tnp1* and *Tnp2* expression levels were dramatically reduced in $Rfx2^{gt/gt}$ mice compared to $Rfx2^{+/+}$ mice (Fig. 5c,d). These results suggest that the differentiation of spermatids is abnormal before or at the round and elongating spermatid

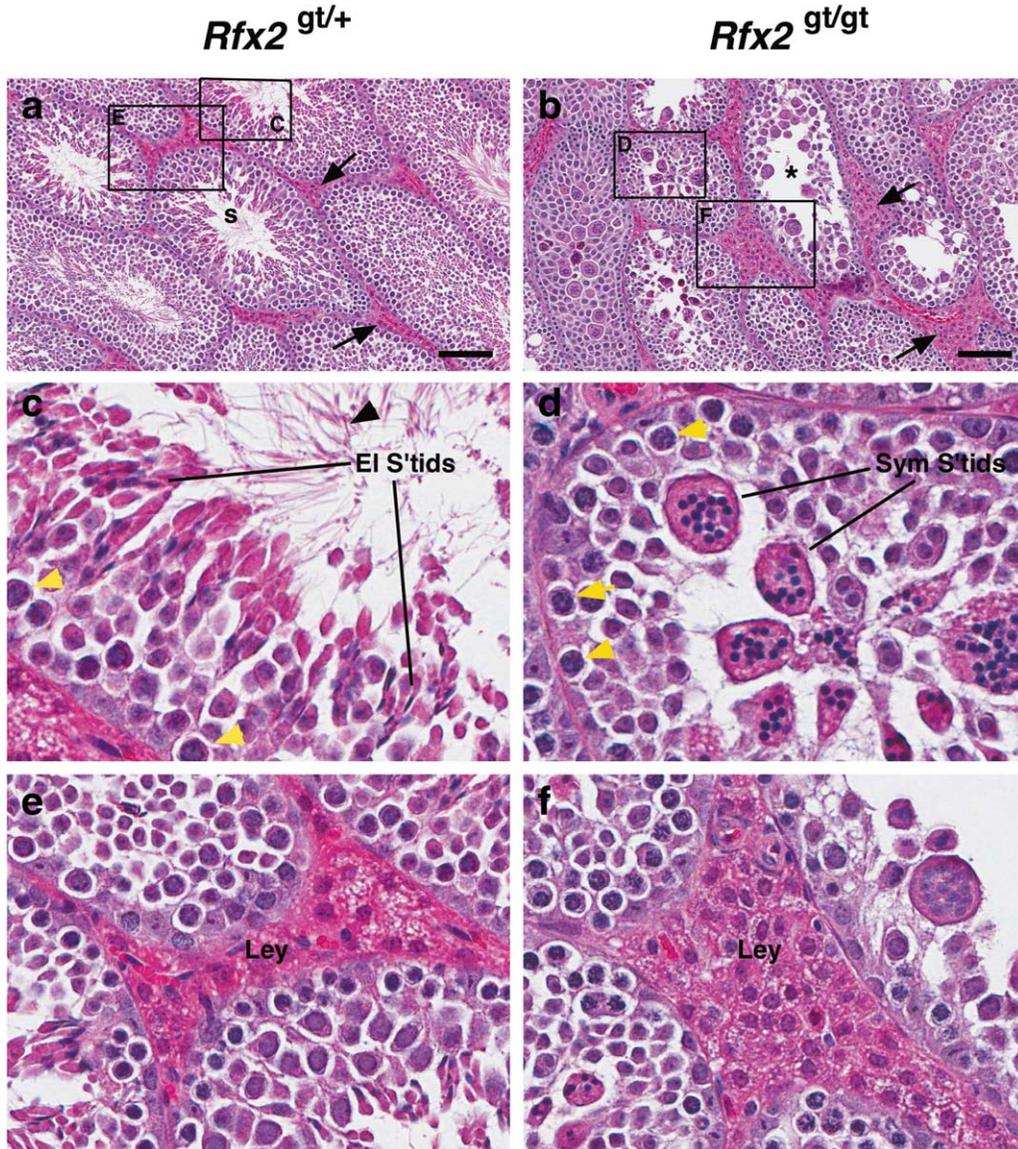


FIG. 4. *Rfx2*^{gt/+} and *Rfx2*^{gt/gt} testis histology. Panels A, C, and E correspond to *Rfx2*^{gt/+}. Panels B, D, and F correspond to *Rfx2*^{gt/gt}. The boxed areas in panels A and B are enlarged in panels C–F. (a,b) Spermatozoa (s) are present in the lumen of seminiferous tubules from *Rfx2*^{gt/+} mice but are absent in *Rfx2*^{gt/gt} testis (*). The arrows point to the interstitial space containing Leydig cells, which appear enlarged in *Rfx2*^{gt/gt} mice compared to *Rfx2*^{gt/+} mice. The bar equals 100 μ m. (c,d) Elongating spermatids (El S'tids) are prominent in the seminiferous tubules of *Rfx2*^{gt/+} testis and give rise to spermatozoa (arrowhead). In *Rfx2*^{gt/gt} testis, elongating spermatids are replaced by symplasts of spermatids (Sym S'tids). Spermatocytes located basally (yellow arrowheads) are present in both *Rfx2*^{gt/+} and *Rfx2*^{gt/gt} testis sections. (e,f) Increased numbers of Leydig cells (Ley) are present in the interstitial space of *Rfx2*^{gt/gt} testis compared with *Rfx2*^{gt/+} testis.

stage. We also examined the expression of two putative *Rfx2* target genes, the variant general transcription factor gene *Alf* (*GTF2a1l*) and the histone *H1t* gene (*Hist1b1t*) (Horvath *et al.*, 2004; Horvath *et al.*, 2009; Wolfe *et al.*, 2004). We found that although the average expression levels of both genes was lower in the KO samples, the standard deviations for both wild-type and KO samples overlapped (Fig. 5e,f).

Our findings indicate that *Rfx2* is not essential for embryonic development in the mouse but is required

for male fertility. The lack of a left-right patterning defect or neural tube closure defect may be because of functional redundancy with other Rfx family members. There are seven Rfx family members and they contain a highly conserved DNA binding domain (Aftab *et al.*, 2008; Emery *et al.*, 1996; Reith *et al.*, 1994; Steimle *et al.*, 1995). *Rfx3* has previously been shown to be expressed in the node (Bonnafe *et al.*, 2004). Consistent with the possibility of overlapping function between *Rfx2* and *Rfx3*, loss of *Rfx3* does not eliminate

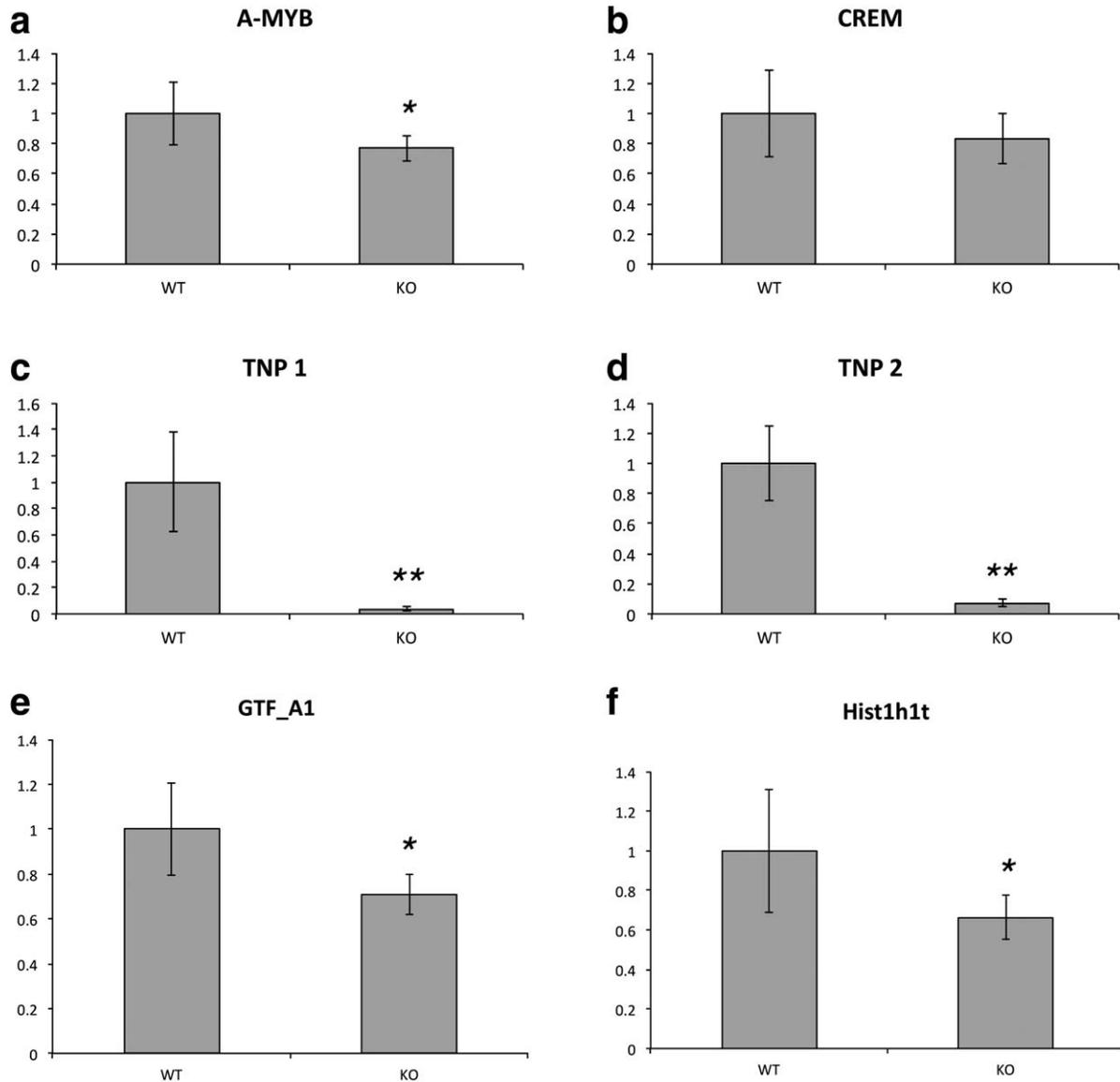


FIG. 5. Analysis of sperm marker gene expression in *Rfx2*^{+/+} and *Rfx2*^{gt/gt} mice by qPCR. **(a,b)** No significant difference in the expression levels of *A-myb* and *CreM* was observed in *Rfx2*^{gt/gt} mice compared to *Rfx2*^{+/+} mice. **(c,d)** Expression of marker genes *Tnp1* and *Tnp2* were downregulated in the testis of *Rfx2*^{gt/gt} mice compared with *Rfx2*^{+/+} mice. **(e,f)** The average expression values of *GTF_A1* (*Gtf2a1*) and *Hist1h1t* were lower in KO samples than in wild-type samples but the standard deviations overlapped. mRNA expression was normalized to *Gapdh* and 9s mRNA for qPCR analysis. **P* < 0.05, ***P* < 0.005.

nodal cilia but rather results in shortening of the cilia and a partially penetrant *situs inversus* phenotype (Bonnafe *et al.*, 2004). In the neural tube, *Rfx4* may substitute for the absence of *Rfx2* as *Rfx4* is uniformly expressed throughout the neuroepithelium before neural tube closure. In mice with a point mutation in the dimerization domain of *Rfx4*, cilia still form but they are stunted and embryos display patterning defects in the ventral spinal cord (Ashique *et al.*, 2009).

The defect in spermatogenesis we observed is consistent with the published data on *Rfx2* expression in the testis (Horvath *et al.*, 2004; Kistler *et al.*, 2009;

Wolfe *et al.*, 2004). *Rfx2* protein is present in pachytene spermatocytes and round spermatids, but it is not present in Sertoli cells or in human Leydig cells (Horvath *et al.*, 2004, <http://www.proteinatlas.org/ENSG00000087903-RFX2/tissue/testis#img>). This suggests that the defect is intrinsic to pachytene spermatocytes and early spermatids. Although *Rfx* factors control the expression of many genes essential for the assembly of ciliary and flagellar axonemes (Ashique *et al.*, 2009; Chung *et al.*, 2012; El Zein *et al.*, 2009), testis histology is largely normal and elongated spermatids lacking flagella can be observed in male-sterile mice

with targeted mutations in axoneme components (Yan, 2009). This suggests that *Rfx2* regulates additional cilia-independent gene programs during spermatid development. Indeed, *Rfx2* controls cell movement and cell remodeling gene programs in addition to ciliogenesis during the maturation of *Xenopus* multi-ciliated cells (MCCs) (Chung *et al.*, 2014). The testis phenotype in *Rfx2* mice resembles the symplastic spermatid phenotype of *Fndc3a*-deficient mice (MacGregor *et al.*, 1990; Obholz *et al.*, 2006). Although there do not appear to be *Rfx2* binding sites in the promoter region of the mouse *Fndc3a* gene (G. MacGregor, personal communication), the related gene, *Fndc3b*, is a direct target of *Rfx2* in MCCs. It is possible therefore, that the genetic program for the maturation of nascent spermatids shares elements with the development of MCCs and that both programs are under *Rfx2* control.

METHODS

Generation of *Rfx2* Gene Trap Mice

Chimeric mice were made by the aggregating clumps of approximately 6–12 ES cells with 8-cell stage CD-1 embryos (Gertsenstein *et al.*, 2010). ES cells were grown in RESGRO culture medium (EMD Millipore) for two passages before aggregation. Embryos were transferred to recipient females and high percentage male chimeras were mated to C57BL/6N females to obtain mice heterozygous for the gene trap allele. Pups were genotyped by PCR. The primers used were: *Rfx2* Fw1 = GGTCTGGAACCAACCCTT CT, *Rfx2* Rv1 = CAGGATTCCCTTGGCAACAGT, and LTRrev2 = CCAATAAACCCTCTT GCAGTTGC. The wild-type allele generates a 267 bp band and the gene trap allele generates a 139 bp band. The PCR cycling parameters were 95°C for 30 seconds, then 35 cycles at 95°C for 10 seconds, 60°C for 30 seconds, 65°C for 1 minute, and a final extension at 65°C for 5 minutes using LongAmp DNA polymerase (New England Biolabs). Mice were maintained on both C57BL/6N and C57BL/6N x CD1 genetic backgrounds. All animal procedures were approved by The University of Texas at Austin Institutional Animal Care and Use Committee. *Rfx2*^{gt/+} sperm on a C57BL/6N background is available from the Texas A&M Institute for Genomic Medicine knockout mouse repository.

Staining for β -Galactosidase (*lacZ*) Activity

Embryos were fixed in 0.2% glutaraldehyde-based fix and stained according to the protocol in *Manipulating the Mouse Embryo* 2nd edition (Hogan *et al.*, 1994).

Testis Histology

Testes were fixed in 4% paraformaldehyde overnight. Paraffin embedding, sectioning and staining was done

by the Histology core at The University of Texas at Austin Dell Pediatric Research Institute as a fee for service.

RT-PCR and qPCR

For RT-PCR, total RNA was isolated from E9.5 embryos using Trizol reagent (Life Technologies). The genotype of each embryo was determined by PCR analysis using DNA isolated from the yolk sac. RNA was reverse transcribed using random primers and M-MuLV reverse transcriptase (New England Biolabs). The primers used for PCR were: exon 8F = GAACCGACTG CAGGAAGACA and exon 12R = TCCACTGTAGGATC GGCTCA. The HPRT primers were CACAGGACTAGAA CACCTGC and GCTGGTGAAAAGGACCTCT (Keller *et al.*, 1993). The PCR cycling parameters were as described above for genotyping.

For qPCR, total RNA was extracted from 3 *Rfx2*^{+/+} and 3 *Rfx2*^{gt/gt} mice on a C57BL/6N background at 2–3 months of age with the Allprep DNA/RNA Mini Kit (Qiagen). The RNA was treated with DNaseI (Invitrogen) and then reverse transcribed using Superscript III (Invitrogen) according to the manufacturer's instructions. A portion of the synthesized cDNA was diluted to generate a standard curve for qPCR reaction. qPCRs were performed using an Applied Biosystems thermocycler. Cycle threshold values were analyzed and normalized to the housekeeping transcripts 9S ribosomal phosphoprotein and *GAPDH* (Ginzinger, 2002; Huggett *et al.*, 2005). The *GAPDH* primers sequences were: *GAPDH* L: GAGTCAACGGATTTGGTTCGT and *GAPDH* R: TTGATT TTGGAGGGATCTCG. The 9S primer sequences were: 9s L: GACTCCGGAACAAACGTGAGGT and 9s R: CTTCA TCTTGCCCTCGTCCA. The sequence of the primers for testis marker genes were: A-myb F = CGTTGGCCGAG ATTGCTAA and A-myb R = CACAATTGCCATCAG GCGAC, Tnp1F = CATGGCATGAGGAGAGGCAA and Tnp1R = GCCCCGTGTTGTTTGAAGAC, Tnp2F = TCG ACACTCACCTGCAAGAC and Tnp2R = ATCCTGGAGT GCGTCACTTG. The *Crem* primers (Cat. No./ID QT01055782), *Gtf2a1l* (Cat./ID: QT00121604,) and *Hist1h1t* (Cat No./ID: QT00250201) were purchased from Qiagen. The *Crem* primers recognize *Crem tau* isoforms (NM_001110856.2, NM_001271504.1, NM_001271505.1, NM_001271506.1). Quantitative PCR reactions and standard curve dilutions were analyzed in triplicate. The cycling conditions were: 94°C for 15 seconds, 60°C for 30 seconds performed for 40 cycles. The specificity of the PCR products was determined by their melting curves. Ct values were extrapolated to a standard curve performed simultaneously with the samples and the data were normalized to the expression of the housekeeping genes *GAPDH* and 9S. Since the Ct values obtained for both housekeeping genes were very similar, a median value of the two

housekeeping Ct values was used to normalize the expression levels of the target genes.

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