REPORT

Translation of the synaptonemal complex component Sycp3 is enhanced in vivo by the germ cell specific regulator Dazl

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ABSTRACT

DAZ-related genes are essential for gametogenesis in diverse metazoa: in human males, a loss of *DAZ* genes is associated with infertility. These genes, expressed only in germ cells, regulate the translation of a yet undefined set of specific transcripts, and loss of function results in numerous defects throughout the mitotic and meiotic process of germ cell development. In a mouse model, absence of the autosomal *Dazl* gene results in a final block at zygotene of meiotic prophase. Sycp3 is also essential for meiosis, specifically for the formation of the synaptonemal complex lateral element with a mouse knockout model displaying a block in meiotic prophase similar to the *Dazl* knock out. Sycp3 was identified as a potential target for translational regulation by Dazl in male mouse germ cells. This was confirmed by both RNA binding and translation assays. In the *Dazl* knockout mouse model, Sycp3 protein levels were decreased, indicating that *Dazl* is required for efficient translation of the Sycp3 mRNA in vivo. Taken together these data support Sycp3 as a biologically relevant target of Dazl-mediated translation in mammals. This suggests that azoospermia associated with a decrease in *DAZ* gene function in humans may in part be a consequence of failure at synapsis caused by reduced levels of SYCP3 protein.

Keywords: translation; gametogenesis; spermatogenesis; RNA binding proteins; infertility

INTRODUCTION

The very highly ordered process of gametogenesis is essential for the faithful transfer of genetic information from one generation to the next. In male mice, this entails mitotic proliferation of germ cells to establish and maintain a population of germline stem cells, followed by meiosis, which results in a reduction from diploid to haploid chromosome complement. Finally, cells undergo a differentiation process known as spermiogenesis to generate mature, motile sperm.

The control of developmental processes by post-transcriptional regulation, in the form of changes in mRNA stability, localization, or translation, is well documented. Germ cell development relies heavily on such post-transcriptional regulation, for example, in the regulation of maternal

mRNAs during oocyte maturation and for translation of stored transcripts during spermiogenesis, a time of transcriptional silencing. This regulation utilizes a complex network of interactions between RNA binding proteins and regulatory sequences within the mRNA, usually located in the UTRs (for review, see Braun 1998; Gray and Wickens 1998; Kuersten and Goodwin 2003; de Moor et al. 2005; Ehrmann and Elliott 2005; Piccioni et al. 2005).

The deleted in azoospermia (*DAZ*) family of germ cell specific translational regulators is required for mitotic and meiotic progression in metazoans (Reijo et al. 1995; Eberhart et al. 1996; Ruggiu et al. 1997; Houston and King, 2000; Karashima et al. 2000) and consists of *Dazl* and *Boule* in vertebrates, with additional Y-chromosomal family members, *DAZ* is present in humans and Old World primates. The *DAZ* family genes encode RNA binding proteins that contain a conserved RNA recognition motif (RRM) and at least one copy of a *DAZ* motif that has been implicated in multiple protein–protein interactions (Tsui et al. 2000a; Dai et al. 2001; Moore et al. 2003, 2004). *DAZ* family proteins bind to U-rich sequences in specific

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.465507>.

transcripts and are required for their efficient translation in vivo (Houston et al. 1998; Tsui et al. 2000b; Venables et al. 2001; Jiao et al. 2002; Maegawa et al. 2002; Collier et al. 2005; Reynolds et al. 2005; Otori et al. 2006). They have been shown to act at the level of translation initiation by directly recruiting a translation initiation factor, polyA binding protein (PABP), to the mRNA through interaction with the 3' UTR of the message (Collier et al. 2005).

In addition to this interaction with PABP, DAZ family proteins also interact with a number of other proteins, including DAZAP1, DAZAP2, PUM2, hQK3, DZIP1, DZIP2, DZIP3, and Dynein light chain, many of which are RNA binding proteins as well as being capable of forming hetero- and homodimers with other DAZ family members (Ruggiu and Cooke 2000; Tsui et al. 2000a; Xu et al. 2001; Moore et al. 2003, 2004; Urano et al. 2005; Lee et al. 2006). The functional significance of many of these interactions remains to be clarified in vivo, although it seems probable that interactions with different proteins will have differing effects on post-transcription regulation of mRNAs, e.g., an interaction with PABP promotes translation, interaction with Pum2 and DAZAP1 (Moore et al. 2003; Urano et al. 2005; Morton et al. 2006) may be involved in translational repression, and interactions with dynein light chain may be required for specific localization of messages (Tsui et al. 2000a; Lee et al. 2006). It is possible that as specific translational requirements change throughout germ cell development, so, too, do the components of the complexes containing *Dazl*.

Several studies have been carried out to identify the RNA targets of Dazl proteins, although few have been able to address the biological significance of those interactions. It is clear, however, that there is a high level of binding specificity to particular RNA sequences (Venables et al. 2001; Jiao et al. 2002; Maegawa et al. 2002; Fox et al. 2005; Reynolds et al. 2005).

A correlation between loss of the Y-chromosomal DAZ genes and infertility in humans has been well established (Reijo et al. 1995). Associations between loss of the other family members and human fertility levels in both males and females have also been suggested (Teng et al. 2002; Luetjens et al. 2004; Tung et al. 2006a,b). Male mice homozygous null for *Dazl* show a range of germ cell specific phenotypes, the precise nature of which are dependent on mouse strain background. This includes the loss of pre-meiotic germ cells before birth, a failure in the transition between A_{aligned} and A₁ spermatogonia, and a final block in meiosis at zygotene of meiotic prophase I (Ruggiu et al. 1997; Schrans-Stassen et al. 2001; Saunders et al. 2003; Lin and Page 2005).

A block in meiotic progression at zygotene is reminiscent of the *Sycp3* null phenotype in male mice (Yuan et al. 2000). Synapsis of homologous chromosomes during zygotene and pachytene of meiotic prophase is essential for the efficient pairing, condensation, and recombination that

must occur for meiosis to progress. This is reliant on the formation of an extensive protein complex, the synaptonemal complex, which holds homologous chromosomes together throughout prophase I. Synaptonemal complex formation begins with the formation of a lateral/axial element containing, among others, the proteins SYCP2 and SYCP3 during leptotene and is followed by the formation of the central element containing SYCE1, SYCE2/CESC1, and TEX12 and the transverse filaments containing SYCP1 during zygotene and pachytene (Meuwissen et al. 1992; Dobson et al. 1994; Lammers et al. 1994; Offenberger et al. 1998; Costa et al. 2005; Hamer et al. 2006).

As for the DAZ family genes, loss of function of synaptonemal complex components in humans has been associated with infertility (Miyamoto et al. 2003; Judis et al. 2004; Sun et al. 2005; Topping et al. 2006). We recently identified the *Sycp3* transcript as a potential target for translational regulation by mouse *Dazl*. Not only was the *Sycp3* transcript found to be associated in vivo with *Dazl* protein in UV cross-linking immunoprecipitation experiments, but the 3' UTR of this mRNA also contains a conserved consensus sequence for *Dazl* binding (Reynolds et al. 2005). Here, we show that *Dazl* protein interacts in vitro with the 3' UTR of *Sycp3* and stimulates its translation in vivo, in a manner that is dependent on the presence of this conserved *Dazl* binding sequence. Furthermore, in *Dazl* null animals, protein levels of *Sycp3* were much reduced. This demonstrates that *Dazl* is required for the efficient translation of *Sycp3* in vivo. We suggest that specific features of the *Dazl* null phenotype may be explained by a failure to achieve adequate *Sycp3* protein levels, an observation that may be extended to the azoospermic phenotype in men lacking DAZ gene function. *Sycp3* is therefore only the second mammalian target for *Dazl*-mediated translation shown to be biologically relevant in vivo.

RESULTS

It has been shown previously that endogenous *Dazl* protein associates with *Sycp3* mRNA in mouse testis (Reynolds et al. 2005). The 3' UTR of *Sycp3* contains a consensus sequence for *Dazl* binding ($U_{2-10}[G/C]U_{2-10}$) that is conserved in the 3' UTR of rat *Sycp3* (Fig. 1A; Reynolds et al. 2005). Given the similarity in phenotype of animals null for either *Dazl* or *Sycp3*, we investigated this interaction in vitro.

Binding was assessed by mixing biotinylated RNA corresponding to the 3' UTR of *Sycp3* with a variety of recombinant *Dazl* proteins in rodent testis homogenate. Testis homogenate was used to mimic endogenous competition conditions for the interaction assay. RNA was isolated using Streptavidin-linked magnetic beads, and the presence or absence of recombinant *Dazl* protein was determined by Western blotting. In this assay, the wild-type *Sycp3* 3' UTR sequence bound equally well to both full-length T7-tagged

also established that binding was direct and independent of other proteins (Fig. 1D).

Dazl has been shown to stimulate translation at the level of initiation of translation when bound to the 3' UTR of mRNAs (Collier et al. 2005; Reynolds et al. 2005). A biologically relevant interaction might thus be expected to result in stimulation of translation (Collier et al. 2005; Reynolds et al. 2005). To test this we used a *Xenopus* oocyte based assay to establish the role of Dazl protein in translation. A fourfold increase in luciferase expression was seen when the wild-type *Sycp3* 3' UTR was fused to the 3' end of the luciferase reporter in the presence of Dazl protein, relative to an unrelated RNA binding protein, U1A. No increase in the expression of the internal control β -galactosidase mRNA or a luciferase mRNA containing an unrelated 3' UTR (data not shown; Fig. 2A) was observed, demonstrating specificity of the effect. Importantly, reporter mRNA levels remain unchanged over the course of the assay, showing that the increase in luciferase activity is a direct result of an increase in translation rather than changes in the stability of the RNA (Fig. 2B). To test whether the Dazl consensus sequence was required for this effect, the mutant *Sycp3* UTR in Figure 1 was appended to the luciferase mRNA. No such increase in translation was seen when the *Sycp3* 3' UTR containing mutations in the Dazl binding site was used (Fig. 2C), establishing a direct link between Dazl binding and translational stimulation.

In order to definitively show that this interaction is of biological significance, Sycp3 protein levels in *Dazl* null mice were measured. In male mice, the first wave of spermatogenesis proceeds synchronously, starting at around day 7 after birth and taking ~35 d to complete (Goetz et al. 1984). Entry into and progression through meiosis can therefore be studied by examining testes from animals in the first weeks after birth. However, in an inbred C57Bl/6 strain background, *Dazl* null mice lose germ cells embryonically, at E13.5, and few or no germ cells can be detected after birth (Fig. 3A; Lin and Page 2005). This is before significant levels of Sycp3 protein are present in wild-type animals.

Dazl null male mice with outbred genetic backgrounds are born with morphologically normal germ cells. Progression through meiosis has been observed up until zygotene of meiotic prophase I (Ruggiu et al. 1997; Saunders et al. 2003). In order to observe early meiotic processes in *Dazl* null animals, we crossed C57Bl/6 *Dazl*^{+/-} males with CD1 *Dazl*^{+/+} females and intercrossed the resultant progeny to produce *Dazl*^{-/-} animals with a reproducible, outbred genetic background.

The germ cell complement was assessed by staining with an antibody directed against the germ cell marker, GCNA (Enders and May 1994). In the outbred background, germ cells persisted after birth in *Dazl* null male mice, although there was considerable variation between individual animals with respect to the number of tubules containing

FIGURE 2. (A) Mouse *Dazl* stimulates translation of a luciferase reporter mRNA relative to an unrelated RNA binding protein, U1A, in the presence of *Sycp3* 3' UTR but not the unrelated 3' UTR of Dengue virus (DV). Neither of the luciferase reporter mRNAs is adenylated. (B) Mouse *Dazl* does not affect mRNA stability. Relative numbers of molecules of luciferase normalized to β -galactosidase cDNA are shown in the presence or absence of mouse *Dazl* at time points zero ($T = 0$) and 16 h ($T = 16$) postinjection and after RNA extraction and reverse transcription. (C) Consensus *Dazl* binding sites are required for *Sycp3* translational regulation. *Dazl* stimulates the translation of luciferase mRNA with the *Sycp3* wild-type but not mutant 3' UTR. For (A) and (C) luciferase values are normalized to a co-injected β -galactosidase mRNA and normalized luciferase activity is plotted, with a value of U1A set to one. Error bars indicate the standard error of the mean.

germ cells. This level of variability reflects the heterogeneous genetic background (Fig. 3B).

In all *Dazl*^{+/+} animals examined at each age, 100% of the tubules contained a full complement of GCNA-positive cells (data not shown). In *Dazl*^{-/-} mice, GCNA positive

cells were detected as late as 3 weeks after birth although normal meiotic progression was not apparent after 7 d post-partum (pp), as assessed by the morphology and position of these GCNA positive cells within the testis tubules (Fig. 3B,C). This is consistent with previous reports of a block at the leptotene/zygotene transition of meiotic prophase I in the absence of *Dazl* (Saunders et al. 2003).

Even in this outbred background there were generally fewer germ cells present in *Dazl* null animals when compared to wild type. Because of the large variation in germ

FIGURE 3. (A) Germ cells survive beyond birth in male *Dazl* null animals with an outbred genetic background (C57Bl/6 × CD1) but not inbred C57Bl/6. Testes were taken at 1 d post partum and stained with anti-Mvh antibody to detect germ cells (red) and counterstained with DAPI (blue). (B) Germ cell survival in outbred *Dazl* null male mice. Testes were taken at varying times after birth, fixed, and stained with anti-GCNA antibody. The percentage of tubules present containing GCNA-positive cells (brown staining) was determined for individual animals. (C) Representative images showing GCNA-positive cells in *Dazl*^{+/+} and *Dazl*^{-/-} animals with C57Bl/6 × CD1 genetic background. Meiotic progression appears normal up until 7 d pp for those germ cells that remain in the null animals. From 10 d pp onward, GCNA-positive cells remain at edges of tubules and/or appear apoptotic, indicating that progression beyond this stage does not occur at any appreciable rate.

cell numbers, measurements were made on a cell-by-cell basis for individual animals in order to compare absolute protein levels between genotypes using quantitative microscopy with identical staining, exposure, and capture conditions.

We were able to select animals at 7 d pp with a normal or close to normal germ cell complement for quantitative immunofluorescence. The amount of Sycp3 protein present in each cell was measured relative to phosphorylated H2AX (γ H2AX). The localization pattern of γ H2AX changes as cells progress through meiotic prophase, enabling only germ cells at equivalent stages of meiosis to be included in the analysis (Fig. 4). This time point was chosen because morphologically normal germ cells were present in most of the *Dazl* null animals tested. We therefore concluded that those germ cells present in the knockout animals were equivalent to those of wild-type animals and therefore

could be compared directly. It is also around the time when a general cessation of normal meiotic progression was observed, and we postulated that more marked changes in protein levels might be seen as a consequence.

Rather than universal lack of Sycp3 protein in *Dazl* null animals, levels varied between cells. Some cells had apparently wild-type levels of Sycp3 protein, while other, neighboring cells contained barely detectable amounts (Fig. 4). In samples from wild-type animals, no cells were detected that were positive for γ H2AX but negative for Sycp3. The variable protein levels in *Dazl* null animals mimic the situation for Mvh protein in testes of *Dazl* null mice, the only other mammalian *Dazl* target that has so far been characterized in vivo (Reynolds et al. 2005).

For three individual *Dazl* null animals examined, average levels of Sycp3 protein in germ cells for individual animals ranged from 55% of wild-type levels to 11%, relative to γ H2AX (Fig. 4B), consistent with *DAZL* enhancing rather than being an absolute requirement for the translation of these mRNAs. Levels of Sycp3 protein in wild-type animals remained comparatively constant from animal to animal. While we do not exclude the possibility that γ H2AX itself is translationally regulated by *Dazl*, these data indirectly suggest that it is not, or that if it is, regulation is not important until later in meiosis.

The same trend was seen when measurements were made both at 10 d pp and when using GCNA as a reference for germ cell protein levels (data not shown). These data confirm that in the absence of *Dazl* protein in vivo levels of Sycp3 protein are reduced and support the hypothesis that *Dazl* mediates Sycp3 translation.

DISCUSSION

This study provides compelling evidence that Sycp3 is an in vivo target of *Dazl*-mediated translation in the male germline. *Dazl* null mice exhibit multiple meiotic phenotypes. A failure in synaptonemal complex formation caused by inadequate levels of Sycp3 protein is likely to contribute to one of these specific defects. In the absence of Sycp3 protein, female mice exhibit defective chromosome segregation, resulting in aneuploidy (Yuan et al. 2002), while a male specific block in meiotic prophase at zygotene is seen (Yuan et al. 2000). One of the defects in *Dazl* null mice is an equivalent arrest at zygotene evident in mice of both sexes. This suggests that there are other mRNAs that require *Dazl* for their efficient translation, particularly during female gametogenesis, which remain to be identified.

Although there are many well-characterized examples of regulated translation of maternal transcripts during oocyte maturation and early embryonic development and of stored transcripts in haploid spermatids, much less is known about the precise nature of *Dazl*-mediated translation in premeiotic and meiotic cells. However, recent work has suggested a role for the translation initiation

FIGURE 4. Quantitative immunofluorescence to measure relative levels of Sycp3 protein in germ cells from *Dazl* $^{+/+}$ and $^{-/-}$ testes 7 d pp. (A) Representative images taken under identical exposure conditions illustrating heterogeneous loss of Sycp3 protein levels in germ cells from *Dazl* null animals ($^{-/-}$). In the merged image, Sycp3 is shown in red and γ H2AX in green. (B) Pixel intensity was measured at the level of individual cells (40 cells per animal) for both Sycp3 staining and γ H2AX, carried out under identical staining and exposure conditions. Sycp3 levels are shown normalized to γ H2AX levels for individual animals. Error bars indicate standard error of the mean.

factor, PABP (Collier et al. 2005) in Dazl-mediated regulation. In this model, Dazl directly recruits PABP to the 3' end of mRNAs. This is reminiscent of the activation of mRNAs by cytoplasmic polyadenylation, a well-characterized mechanism of translational control that is of critical importance during gametogenesis and early embryogenesis (Gray and Wickens 1998). The parallels between poly(A) and Dazl-mediated recruitment of PABP raise the possibility that Dazl is likely to be of particular importance for mRNAs with short poly(A) tails that would not otherwise efficiently recruit PABP (Collier et al. 2005).

In this respect, it is interesting to note that Sycp3 mRNA is stored with a short poly(A) tail of only ~20 nucleotides and undergoes cytoplasmic polyadenylation (Tay and Richter, 2001), with variation in polyadenylation perhaps contributing to the observed variation in SYCP3 levels (Fig. 4). This raises the possibility that Sycp3 may require both cytoplasmic polyadenylation and Dazl-mediated recruitment of PABP for its full level of translational activation, an idea that is supported by the finding that adenylated reporter mRNAs bound by Dazl are translated to a higher level than those lacking a poly(A) tail (Collier et al. 2005). This idea is also in keeping with the lower level of Sycp3 translation in both CPEB and *Dazl* male knockout mice. However, the binding sites for Dazl and CPEB1 in the Sycp3 3' UTR overlap (Fig. 1A), making it questionable whether the two proteins would be able to bind to the 3' UTR simultaneously, although a physical interaction between DAZ-1 and CPB-3 has been reported in *Caenorhabditis elegans* (Hasegawa et al. 2006). By immunofluorescence, we were able to detect only a low level of colocalization between Dazl and CPEB1 proteins in the cytoplasm of mouse spermatocytes and have been unable to demonstrate a physical interaction between the two by coimmunoprecipitation (data not shown). Thus it is possible that Dazl and CPEB1 act sequentially to control Sycp3 translation in vivo. Moreover, it should be remembered that CPEB, like Pumilio, is also involved in translational repression and that the precise composition of Dazl complexes may determine whether the translation of a particular message is enhanced or repressed. Furthermore, it is possible that Dazl-mediated regulation of some mRNAs may result in direct or indirect changes in RNA stability or even localization.

In the absence of Dazl in the mouse male germline, translation of target transcripts continues at a low level since proteins such as Mvh and Sycp3 are still produced, albeit at reduced levels when compared to wild type (this study; Reynolds et al. 2005). It seems that only upon failure to reach a threshold level of a particular protein, or when a number of proteins are present at insufficient levels, do cells arrest and/or die. This would account for the complex phenotype seen in *Dazl* null mice. Moreover, survival of *Dazl* null germ cells was enhanced in an outbred background, indicating that there are modifiers of the phenotype, perhaps in the form of other DAZ family members,

other RNA binding proteins, or more general translational regulators. Identification of these would provide valuable insight into the mechanism of action of DAZ family proteins.

MATERIALS AND METHODS

RNA protein interaction assays

Assays were carried out as described previously (Reynolds et al. 2005). Bacterially produced, GST-tagged, truncated Dazl proteins (wild type and point mutated) have been described elsewhere (Reynolds et al. 2005), while T7-tagged, full-length Dazl was produced by transfecting the plasmid pCGDazl into 293T cells according to Cazalla et al. (2005). The full-length Sycp3 3' UTR was cloned by PCR from adult mouse testis cDNA into pGEM-T Easy (Promega) to create pGEMTEasySycp3 3' UTR, which was sequenced fully. Mutations were introduced by PCR, products cloned into pGEM-T Easy, and inserts sequenced fully. Transcription from linearized plasmid was carried out using either T7 or SP6 RNA Polymerase-Plus (Ambion) as appropriate. Oligonucleotide sequences used were designed to the Sycp3 3' UTR sequence NM_011517 as follows:

Forward: TGAGTCTTTGAAGAAAGAACTTGA and either
WT-reverse: AAATTTAAATCATCTTTATTGACACA or
mutant-reverse: AAATTTAAATCATCTTTATTGACACAATCGTG
GAGAGGGAGGCTATTGGGGAGGGCGGGA.

Proteins were detected with antibodies directed against either GST (Sigma) or T7 (Novagen). Filter binding assays were performed as described by Reynolds et al. (2005).

Translation assays

Translation assays using mRNAs microinjected into stage VI *Xenopus* oocytes were carried out as described using the plasmids pUC19LUC-Scp3wt and pUC19LUC-Scp3mut, which were created by inserting the 3' UTR of Sycp3 as a *Sall*–*HindIII* fragment into the vector pUC19LUC (Reynolds et al. 2005). pUC19LucDV(3' UTR) is described elsewhere (Reynolds et al. 2005).

mRNA stability assays

Total RNA was extracted from pools of oocytes immediately and 16 h following injection of the reporter mRNAs using Tri-Reagent (Sigma) as per the manufacturer's instructions. RNAs were reverse transcribed using an Omniscript RT kit (Qiagen) using reverse primers specific for either the luciferase (AGCGTTTCCCGGTA TCCA) or β -galactosidase reporter (CTGATTTGTGTGGTCCGT TTGT).

Quantitative PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The number of molecules of each reporter cDNA per sample was measured by incorporation of SYBR green into PCR products using SYBR GREEN PCR Master Mix (Applied Biosystems) and the following primers: Luc 5' (GGCGCGGTCCGGTAAAGTT) and Luc 3' (AGCG TTTTCCCGGTATCCA) for luciferase, and Gal 5' (TCAGGATAT GTGGCGGATGA) and Gal 3' (CTGATTTGTGTGGTCCGTTTGT).

for β -galactosidase. Data analysis was performed with the ABI Prism SDS2.1 software (Applied Biosystems), and standard curves generated from luciferase and β -galactosidase DNA standards were used to determine number of molecules in the test samples. All samples were analyzed in triplicate.

Immunofluorescence and quantification of protein levels

Antibodies used were rat anti-GCNA (a gift from G.C. Enders [Enders and May 1994]), rabbit anti-Sycp3 (a gift from C. Heyting [Lammers et al. 1994]), rabbit anti-Mvh (Abcam), and mouse anti-phosphohistone H2A.X, clone JBW301 (Upstate).

Imaging was carried out as described previously using constant exposure times for each image. Subsequent quantification was performed using IPLab Spectrum software (Scanalytics Corp.) (Reynolds et al. 2005).

Animals and tissue collection

Dazl^{Tm1hgu/Tm1hgu} mice (Ruggiu et al. 1997) were housed under standard conditions and fed ad libitum. Generation and breeding of mice was covered by Home Office Project Licence 60 3067 and was approved by institutional ethics committees.

C57Bl/6 inbred mice used were from a line that had been backcrossed to pure C57Bl/6 mice a minimum of eight times.

Outbred lines were established by mating C57Bl/6 Dazl^{+/-} male mice with CD1 females and intercrossing the resultant Dazl^{+/-} animals to produce homozygous ^{+/+} and ^{-/-} animals.

Testes were recovered from mice at varying times after birth (with the day of birth designated 0.5 d post-partum) and fixed in 4% PFA. Tail tips were collected at the time of death for genotyping by PCR. Testes were fixed in 4% PFA for 2–6 h depending on size before dehydration and mounting in paraffin wax. Sections (0.6 μ m) were cut and processed using standard methods for immunofluorescence or immunohistochemistry.

ACKNOWLEDGMENTS

We thank members of the Cooke laboratory, past and present, for useful discussions and critical reading of the manuscript. We also thank Mary Taggart and Rob Speed for technical assistance, Janice Young for help with animal husbandry, Susan Lynas and William Richardson for technical assistance with *Xenopus*, Paul Perry for help with microscopy, and Sandy Bruce for figure production. Professor G.C. Enders and Professor C. Heyting kindly provided antibodies. This work was funded by the U.K. Medical Research Council. B.C. and N.K.G. are funded by a Wellcome Trust Project grant and an MRC Senior Fellowship awarded to N.K.G.

Received January 17, 2007; accepted April 24, 2007.

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RNA 2007 13: 974-981 originally published online May 25, 2007

Access the most recent version at doi:[10.1261/rna.465507](https://doi.org/10.1261/rna.465507)

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