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Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice

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Abstract

Genes of the *DAZ* family play critical roles in germ cell development in mammals and other animals. In mice, *Dazl* mRNA is first observed at embryonic day 11.5 (E11.5), but previous studies using *Dazl*-deficient mice of mixed genetic background have largely emphasized postnatal spermatogenic defects. Using an inbred C57BL/6 background, we show that *Dazl* is required for embryonic development and survival of XY germ cells. By E14.5, expression of germ cell markers (*Mvh*, *Oct4*, *Dppa3/Stella*, GCNA and MVH protein) was reduced in XY *Dazl*−/− gonads. By E15.5, most remaining germ cells in XY *Dazl*−/− embryos exhibited apoptotic morphology, and XY *Dazl*−/− gonads contained increased numbers of TUNEL-positive cells. The rare XY *Dazl*−/− germ cells that persisted until birth maintained a nuclear morphology that resembled that of wildtype germ cells at E12.5–E13.5, a critical developmental period when XY germ cells lose pluripotency and commit to a spermatogonial fate. We propose that *Dazl* is required as early as E12.5–E13.5, shortly after its expression is first detected, and that inbred *Dazl*−/− mice of C57BL/6 background provide a reproducible standard for exploring *Dazl*'s roles in embryonic germ cell development.

Keywords: *Dazl*; *DAZ*; Embryonic; *Dazl* knockout; Pluripotency; Germ cell; C57BL/6

Introduction

Genes in the *Deleted in Azoospermia* (*DAZ*) family are expressed specifically in germ cells and are required for fertility in flies, worms, fish, frogs and mammals (Eberhart et al., 1996; Hashimoto et al., 2004; Houston and King, 2000; Karashima et al., 2000; Reijo et al., 1995; Ruggiu et al., 1997; Xu et al., 2001). *DAZ* was first discovered in studies of the human Y chromosome, in a region whose deletion results in spermatogenic failure (Reijo et al., 1995). Studies of mouse *Dazl* have focused primarily on postnatal roles in spermatogenesis, while embryonic functions in XY germ cells remain largely unexplored (Maratou et al., 2004; Ruggiu et al., 1997; Saunders et al., 2003; Schrans-Stassen et al., 2001; Slee et al., 1999; Vogel et al., 2002). However, disruptions of *Dazl* homologs in other vertebrates, including *Xenopus* and zebrafish, suggest that *Dazl* might be crucial to the development and survival of embryonic germ cells (Hashimoto et al., 2004; Houston and King, 2000; Maegawa et al., 1999).

In mice, primordial germ cells invade the genital ridge by E10.5, and XY germ cells cease mitotic divisions between E13.5 and E16.5 (Nagano et al., 2000). Mouse *Dazl* is first expressed at E11.5, in post-migratory germ cells (Seligman and Page, 1998). However, studies of mice of mixed strain background revealed no defects in XY *Dazl*−/− germ cells at E15.5 (Ruggiu et al., 1997). The reported timing of postnatal defects and death in XY *Dazl*−/− germ cells has varied, with significant numbers of germ cells noted weeks after birth (Schrans-Stassen et al., 2001; Saunders et al., 2003). It is difficult to gauge how much of the observed variability in postnatal germ cell numbers and development is an inherent feature of *Dazl* deficiency and how much is due to differences

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in genetic modifier loci that differ among strains (Saunders et al., 2003). To establish a reproducible standard for examining *Dazl* functions, we backcrossed the published knockout allele ($Dazl^{TM1/Hgu}$) onto the C57BL/6 background. Our experiments indicated that *Dazl* is required for XY germ cell development and survival as early as E12.5–E13.5.

Materials and methods

Backcrossing $Dazl^{TM1/Hgu}$ onto C57BL/6 strain background

Mice carrying the $Dazl^{TM1/Hgu}$ allele (Ruggiu et al., 1997) were generously provided by Howard Cooke, MRC Human Genetics Unit, Edinburgh, UK. We crossed $Dazl^{TM1/Hgu}$/+ mice to C57BL/6 mice (Taconic Farms Inc., Germantown, NY). All experiments were carried out on mice backcrossed to C57BL/6 between 7 and 11 generations, when 99.2% to 99.9% of the genome is expected to be of C57BL/6 origin; all Y chromosomes and mitochondria are of C57BL/6 origin.

Embryonic gonad collection and PCR-based genotyping

Timed matings were performed. Noon on the day a vaginal plug was found was designated as E0.5. Collection and processing of embryos for in situ hybridization, as well as PCR sexing of E11.5 embryos, were carried out as published (Menke et al., 2003). Genotypes were defined by PCR as previously described (Ruggiu et al., 1997).

Histology, immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis

For histological studies, gonadal tissues were fixed in Bouins solution overnight at 4°C. For immunohistochemistry and TUNEL analysis, gonadal tissues were fixed in 4% paraformaldehyde overnight at 4°C or for 4 h at room temperature. Fixed tissues were embedded in paraffin and sectioned. Slides for histological examination were stained with hematoxylin and eosin. Just before use, slides for immunohistochemistry were dewaxed, rehydrated, and autoclaved in 10 mM sodium citrate for 5 min at about 121°C. Rat anti-germ cell nuclear antigen (GCNA), a gift from George Enders, University of Kansas, Kansas City, was used as previously described (Enders and May, 1994). Rabbit anti-phosphohistone H3 (PH3, Upstate Biotech. #07-145) was used at a 1:300 dilution. FITC-conjugated anti-rat secondary antibody and Texas Red-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.) were used at 1:40 and 1:200 dilutions, respectively. TUNEL analysis was carried out using the FragEL™ DNA Fragmentation Detection Kit, Fluorescent (EMD Biosciences) according to the manufacturer's instructions. Microscopic examination and image capture were conducted at the W. M. Keck Foundation Biological Imaging Facility at the Whitehead Institute.

In situ hybridization

In situ hybridization on embryonic gonads and subsequent image capture were carried out as previously described (Menke et al., 2003; Wilkinson and Nieto, 1993). For whole-mount in situ hybridization, *Dazl*+/+ and *Dazl*−/− gonads were co-processed in single tubes to exclude tube-to-tube variation as a source of differential staining. Digoxigenin riboprobes were generated as previously described (Menke and Page, 2002; Menke et al., 2003). The *Oct-4* probe was described previously (Menke et al., 2003). The *Dazl* probe was a gift from Min Wu, Whitehead Institute. PCR primers used to generate *Dppa3/Stella* and *Mouse vasa homologue* (*Mvh)* probes were designed using Primer3 software (Rozen and Skaletsky, 2000). Below are descriptions of previously unpublished probes.

Gene	Forward primer for PCR	Reverse primer for PCR	Probe size
Dppa3/Stella	TTCCGAGCTAGCTTTTGAGG	CTGGATCGTTGTGCATCCTA	641 bases
Mvh	CACCGGCAATTTTGACTTTT	AGCAACAAGAACTGGGCACT	937 bases
Dazl	Full-length *Dazl* cDNA		1.1 kilobase

Results

Dazl mRNA expression begins at E11.5

To determine when *Dazl* is first expressed in embryonic XY C57BL/6 gonads, we performed in situ hybridization with a full-length *Dazl* riboprobe. At E11.5, no *Dazl* mRNA was observed in most XY gonads examined, but faint staining could be seen in some (Fig. 1). By E12.5, *Dazl* mRNA was readily detected in the nascent testis cords of all XY gonads examined. By E13.5, *Dazl* transcripts were abundant in testis cords (Fig. 1).

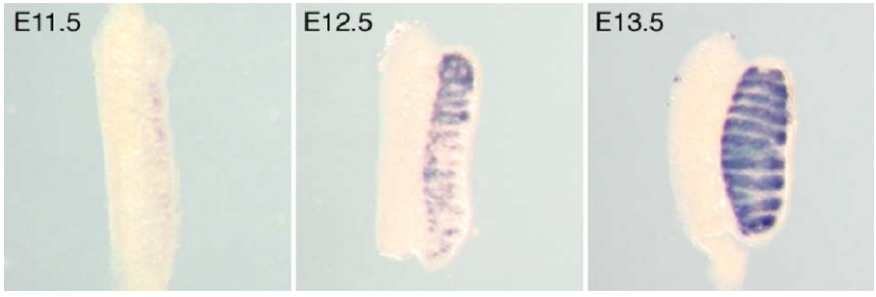

Fig. 1. Whole-mount in situ hybridization of *Dazl* riboprobe to wildtype XY embryonic gonads.

Testes of Dazl−/− neonates contain very few germ cells

All 60 histological sections from testes of two wildtype XY neonates (D0.5) contained multiple germ cells per testis cord, and the chromatin of these germ cells, as expected, was diffused rather than condensed (Figs. 2A, B). (Germ cells were distinguished from somatic cells by their central positions within testis cords and by their large nuclei.) By contrast, of 60 histological sections from testes of two *Dazl−/−* neonates, only two contained identifiable germ cells and then only in two cord cross-sections (Fig. 2C). These germ cells appeared abnormal, with chromatin condensed at the edge of the nucleus (Fig. 2D). As a more sensitive means of detecting germ cells in neonatal testes, we assayed for expression of Mouse Vasa Homologue (MVH), a germ-cell-specific cytoplasmic protein. All ten testes sections examined from three wildtype neonates displayed multiple MVH-positive germ cells in each testis cord; each section contained 100–200 such cells (Fig. 2E). By contrast, of 18 testis sections from six *Dazl−/−* neonates, 12 sections contained no MVH-positive cells, and the others displayed fewer than ten MVH-positive cells (Fig. 2F and Fig. S1 in Appendix A).

Histologic abnormalities appear by E14.5

As we observed virtually no germ cells in XY *Dazl−/−* testes at birth, we next examined the histology of embryonic XY gonads. At E13.5, XY wildtype and *Dazl−/−* gonads could not be distinguished histologically (Figs. 3A, B). At E14.5, germ cells with dark condensed nuclei, typical of apoptotic cells, were observed in XY *Dazl−/−* gonads (two embryos examined; Fig. 3D). Such cells were rarely seen in gonads of wildtype XY littermates (Fig. 3C). At E15.5, many germ cells in XY *Dazl−/−* gonads appeared apoptotic (two embryos examined; Fig. 3F). At E17.5, many testis cords in XY *Dazl−/−* gonads contained only somatic cells, and few cords contained germ cells that did not appear apoptotic (three embryos examined; Fig. 3H).

From E14.5 through birth, the chromatin of wildtype XY germ cells gradually assumes a more diffuse appearance

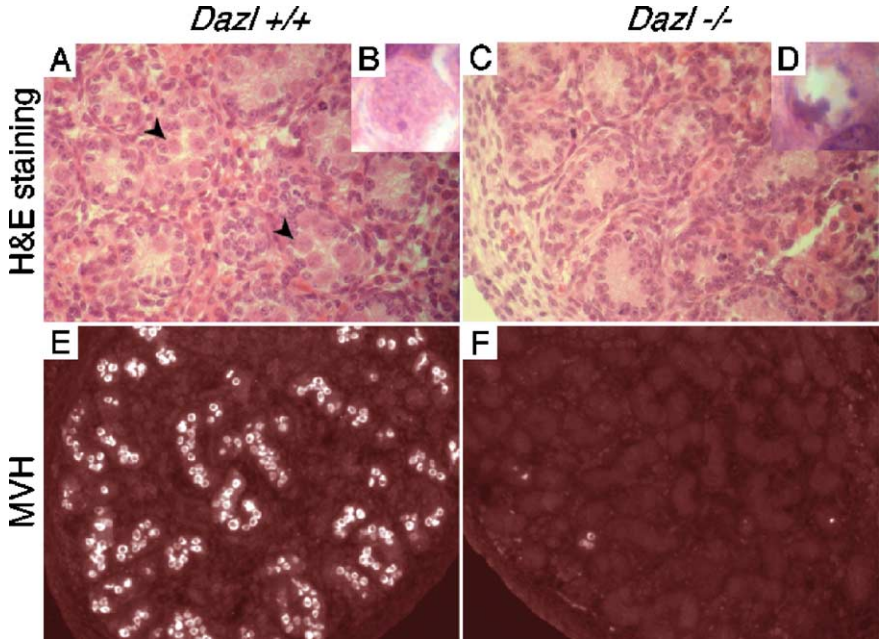

Fig. 2. Sections of neonatal (D0.5) testes from XY *Dazl+/+* (A, E) and *Dazl−/−* (C, F) mice stained with hematoxylin and eosin (A, C, 40× magnification) or with MVH antisera (E, F, 10× magnification). Inserts show high magnification images of a typical D0.5 XY *Dazl+/+* germ cell (B) and a rare D0.5 XY *Dazl−/−* germ cell (D). Arrowheads indicate germ cells. See Fig. S1 in Appendix A for additional sections stained with MVH antisera.

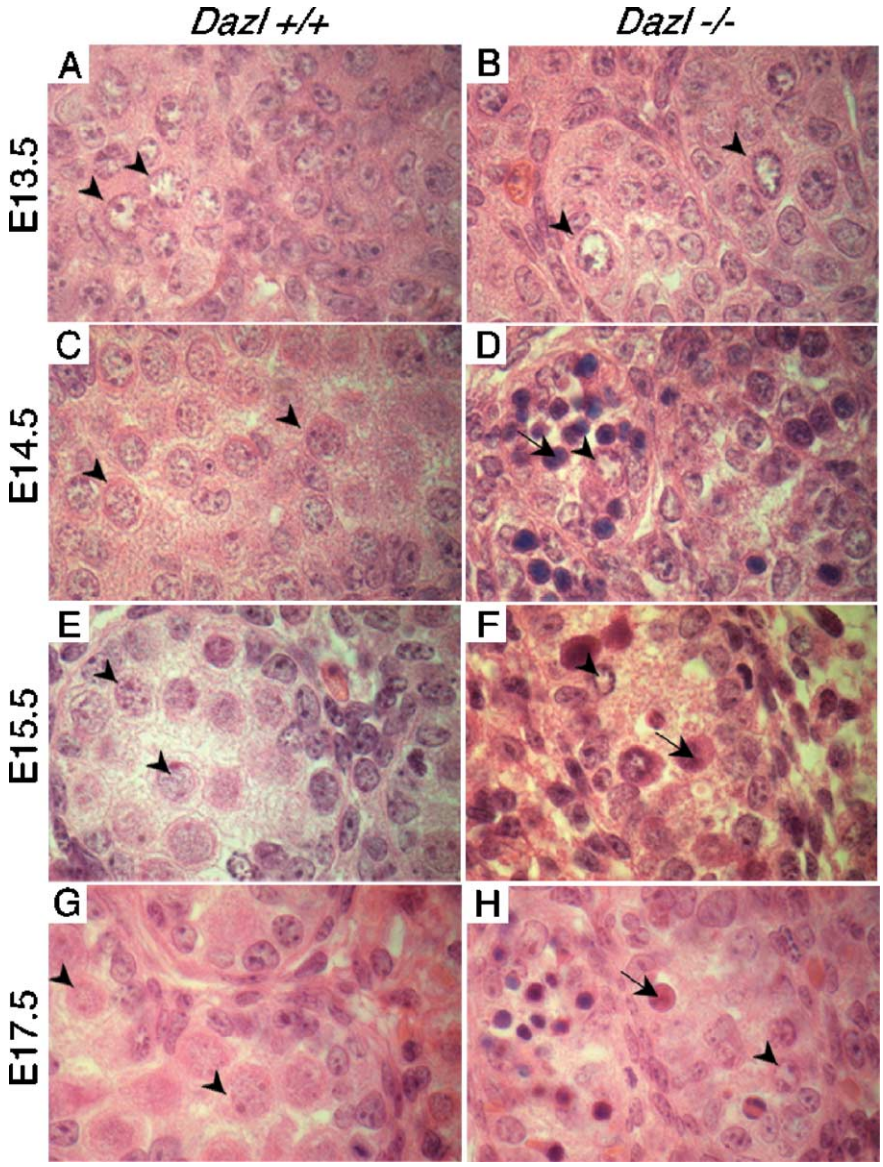

Fig. 3. Hematoxylin and eosin stained sections of embryonic gonads from XY *Dazl*+/+ (A, C, E, G) and *Dazl*−/− (B, D, F, H) littermate mice at E13.5 (A, B), E14.5 (C, D), E15.5 (E, F) and E17.5 (G, H). Arrowheads indicate germ cells. Arrows indicate presumptive apoptotic germ cells.

(Figs. 3C, E, G and Fig. 2B). By contrast, the chromatin of surviving XY *Dazl*−/− germ cells maintains the condensed appearance typical of wildtype germ cells at E12.5–E13.5 (Figs. 3D, F, H and Fig. 2D).

Loss of germ cell markers by E14.5–E15.5

To confirm the embryonic loss of germ cells, we assayed expression of germ-cell-specific markers (*Mvh, Dppa3/Stella*, *Oct4*, GCNA and MVH protein) in XY gonads, both wildtype and *Dazl*−/− (Bowles et al., 2003; Fujiwara et al., 1994; Ovitt and Scholer, 1998; Saitou et al., 2002). At E14.5, in situ hybridization revealed a modest but consistent decrease in the levels of *Mvh*, *Oct4* and *Dppa3/Stella* transcripts in XY *Dazl*−/− gonads compared with littermate controls (Figs. 4A–F). By E15.5, a marked reduction in transcript levels was evident (data not shown). In addition, immunofluorescence studies revealed that both MVH protein expression at E14.5 and GCNA expression at E15.5 were greatly reduced in XY *Dazl*−/− gonads compared to wildtype controls (Figs. 4G–J).

Increased numbers of TUNEL-positive cells in Dazl−/− gonads at E15.5

The presence of apparently apoptotic germ cells led us to assay XY E15.5 gonads for apoptotic nuclei by fluorescent TUNEL analysis (Figs. 5A, B), using DAPI staining to identify testis cords and germ cell nuclei (Figs. 5C, D). We found that the number of TUNEL-positive cells was significantly increased in XY *Dazl*−/− embryonic gonads compared to littermate controls (Figs. 5A, B), despite the

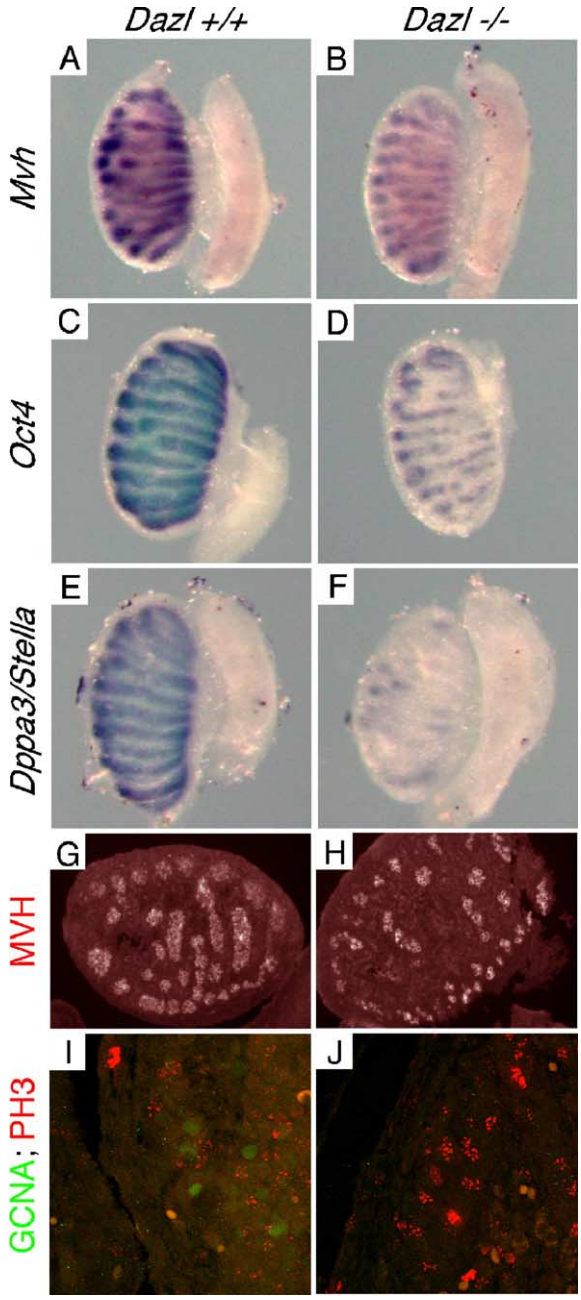

Fig. 4. Staining for germ cell markers in XY *Dazl*+/+ and *Dazl*−/− embryonic gonads. (A–F) Whole-mount in situ hybridization of *Mvh* (A, B), *Oct4* (C, D) or *Dppa3/Stella* (E, F) riboprobes to littermate E14.5 XY *Dazl*+/+ (A, C, E) and *Dazl*−/− (B, D, F) gonads. (G, H) Fluorescent immunohistochemistry reveals MVH-positive germ cells (red) on sections of E14.5 XY *Dazl*+/+ (G) and *Dazl*−/− (H) gonads (10× magnification). (I, J) Fluorescent immunohistochemistry reveals GCNA-positive germ cells (green) and phosphohistone H3-positive (PH3) dividing cells (red) on sections of E15.5 XY *Dazl*+/+ (I) and *Dazl*−/− (J) gonads (60× magnification). In wildtype XY gonads at E15.5, somatic cells are dividing rapidly and hence are PH3-positive, while germ cells are mitotically inactive and PH3-negative. The death of germ cells in *Dazl*−/− gonads effectively increases the proportion of PH3-positive (somatic) cells there.

fact that the number of germ cell nuclei was decreased in XY *Dazl*−/− gonads (Figs. 5C, D). Furthermore, most TUNEL-positive nuclei in XY *Dazl*−/− gonads were found within testis cords (Figs. 5E, F). Thus, the TUNEL analysis confirms that apoptosis accounts for the dramatic loss of germ cells observed in XY *Dazl*−/− embryos.

Discussion

Using an inbred genetic background, we have found that *Dazl* plays an essential role in embryonic development and survival of XY germ cells. The requirement for *Dazl* manifests soon after its transcripts are detected, at about the time when XY germ cells lose pluripotency and commit to a spermatogenic fate.

Our *Dazl* expression study in XY C57BL/6 embryonic gonads is in agreement with published data. The faint in situ hybridization signal that we observed at E11.5 and the stronger signal at E12.5 correspond well with previous data obtained at these time points using RT-PCR and Northern analysis (Seligman and Page, 1998).

In the present study, we observed that, at E15.5, most germ cells in XY *Dazl*−/− gonads appeared apoptotic, and the number of TUNEL-positive cells was elevated in *Dazl*−/− compared to *Dazl*+/− testes. In addition, several germ-cell-specific markers showed reduced expression in XY *Dazl*−/− gonads compared to wildtype littermates at E14.5 and E15.5. In summary, on a C57BL/6 strain background, germ cell loss in XY *Dazl*−/− gonads occurred as early as E14.5–E15.5.

The nuclear morphology of the rare XY *Dazl*−/− germ cells that survived to birth resembled that of wildtype germ cells at E12.5–E13.5, suggesting a time frame for *Dazl*'s embryonic function. We infer that developmental arrest at E12.5–E13.5 precedes germ cell death in XY *Dazl*−/− embryos. Published studies indicate that profound developmental changes take place during this period. This is the last embryonic stage from which EG (embryonic germ) cell lines – which resemble ES cell lines in their pluripotency – can be derived (Labosky et al., 1994). Conversely, only after E12.5 can wildtype embryonic XY germ cells repopulate an adult testis without co-transplanting donor somatic cells (Ohta et al., 2004). *Dazl* function at E12.5–E13.5 thus coincides with the transition of XY germ cells from a pluripotent state to a more differentiated spermatogenic identity. Only two other germ-cell-specific genes, *Mvh* and *Nanos2*, are known to be required at this developmental juncture between completion of germ cell migration (at E10.5) and cessation of mitotic division (at E13.5–E16.5) (Tanaka et al., 2000; Tsuda et al., 2003). The C57BL/6 *Dazl* knockout can now be used to study *Dazl* function in relation to the mechanism by which pluripotent XY germ cells make a transition to a more restricted spermatogenic fate.

Our findings stand in contrast to previous studies, all conducted in mixed genetic backgrounds, that suggest that *Dazl* is essential to XY germ cell development and survival only later in development (Ruggiu et al., 1997; Schrans-Stassen et al., 2001; Saunders et al., 2003; Maratou et al.,

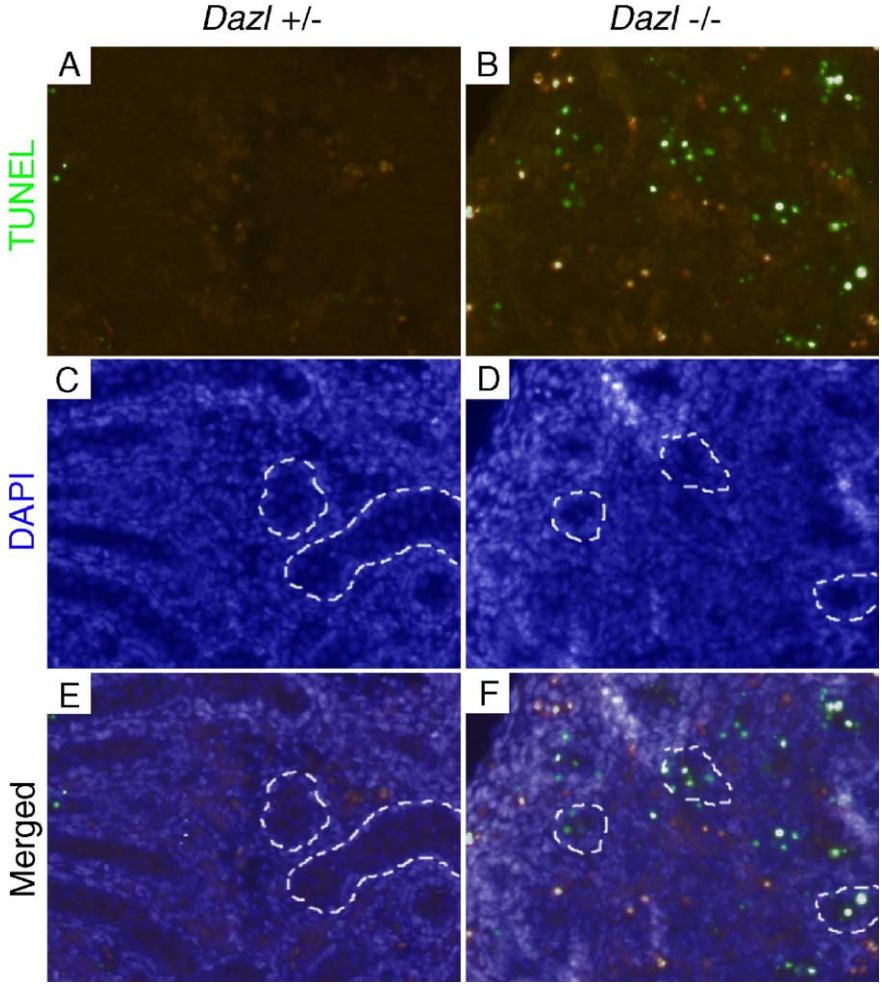

Fig. 5. TUNEL and DAPI staining in E15.5 XY *Dazl*+/− (A, C, E) and *Dazl*−/− (B, D, F) gonad sections. TUNEL-positive cells visualized in green (A, B) and DAPI-positive nuclei in blue (C, D). Merged images in panels (E) and (F). Orange spots arise from non-specific fluorescence (A, B, E, F). Dotted white lines delineate some of the testis cords (C–F).

2004). The earliest reported phenotype on a mixed genetic background is a decrease in germ cell numbers at E19.5 (Ruggiu et al., 1997). Another report characterized the XY *Dazl*−/− phenotype as a failure of postnatal spermatogonial differentiation, with no significant difference between germ cells of wildtype and *Dazl*−/− testes until 9 days after birth (Schrans-Stassen et al., 2001). A third study concluded that the final defect in XY *Dazl*−/− germ cell development is a block in prophase I of the first wave of meiosis, occurring 1 to 2 weeks after birth (Saunders et al., 2003). Most recently, a study comparing RNA expression profiles of postnatal XY *Dazl*−/− and wildtype testes concluded that leptotene-zygotene of meiosis is the most advanced stage attained by XY *Dazl*−/− germ cells (Maratou et al., 2004).

These phenotypic studies of mixed background mice, together with the fact that DAZL protein is present in early spermatocytes, argue that *Dazl* has important functions in postnatal XY germ cell development (Ruggiu et al., 1997). To explore these postnatal functions in greater detail, experiments must be carried out in a reproducible pure-strain background (not yet identified) where XY *Dazl*−/− germ cells survive in the neonate. Alternatively, a conditional *Dazl* allele must be made.

Our finding that *Dazl* is required in XY embryonic germ cells has implications for biochemical studies of the mouse DAZL protein. Previously, searches for mRNA targets of DAZL, a putative RNA-binding protein, have assumed that adult testis is the relevant tissue in which to identify targets (Jiao et al., 2002; Venables et al., 2001). The DAZL protein may bind different mRNAs in embryonic germ cells. Similarly, we suggest that microarray transcription profiling experiments in *Dazl* knockout mice be extended to embryonic XY gonads.

Previously reported variability in the timing of germ cell death in mice of mixed genetic background and the earlier phenotype seen in our present study of C57BL/6 animals indicate that strain background strongly affects the phenotype of XY *Dazl*−/− mice. Published studies using mice of uncertain or mixed genetic background may need to be reinterpreted in this light. In efforts to rescue the phenotype of XY *Dazl*−/− mice with human *DAZ* or *DAZL* transgenes, the human genes were inserted into ES cells from mice of

different mixed backgrounds (Vogel et al., 2002). The degree of rescue observed with human *DAZ* transgenes was variable and appeared to be independent of transgene copy number, insertion site and integrity of the *DAZ* coding region (Vogel et al., 2002). Perhaps some phenotypic effects attributed to the transgenes actually represented the effects of strain-dependent modifiers introduced along with the transgenes.

Sensitivity of a mouse embryonic germ cell defect to strain background is not unique to *Dazl*. Disruption of *Pin1* yielded no such abnormalities prior to backcrossing to C57BL/6, where both XY and XX *Pin1*-deficient embryos displayed poor embryonic germ cell proliferation (Atchison et al., 2003; Spears et al., 2003). In *TrkB* −/− neonatal ovaries, grossly abnormal morphology and reduced follicle numbers were observed in 50% of mixed background mice but in 100% of C57BL/6 mice (Spears et al., 2003). Thus, strain dependence of mouse embryonic germ cell phenotypes may be common. Inbred C57BL/6 *Dazl* knockout mice now provide opportunity to study a newly identified requirement for *Dazl* during a developmental period when crucial changes occur in XY germ cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.06.032.

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