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The mouse *dead-end* gene isoform α is necessary for germ cell and embryonic viability

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Abstract

Inactivation of the *dead-end* (*Dnd1*) gene in the *Ter* mouse strain results in depletion of primordial germ cells (PGCs) so that mice become sterile. However, on the 129 mouse strain background, loss of *Dnd1* also increases testicular germ cell tumor incidence in parallel to PGC depletion. We report that inactivation of *Dnd1* also affects embryonic viability in the 129 strain. Mouse *Dnd1* encodes two protein isoforms, DND1-isoform α (DND1-α) and DND1-isoform β (DND1-β). Using isoform-specific antibodies, we determined DND1-α is expressed in embryos and embryonic gonads whereas DND1-β expression is restricted to germ cells of the adult testis. Our data implicate DND1-α isoform to be necessary for germ cell viability and therefore its loss in *Ter* mice results in PGC depletion, germ cell tumor development and partial embryonic lethality in the 129 strain.

Keywords: Testicular germ cell tumors; *dead-end*; *Dnd1*; Isoform; Antibodies; *Ter*

The 129-*Ter* mouse strain develops testicular germ cell tumors (TGCTs) similar to congenital tumors which occur in the testes of human infants (testicular type I germ cell tumors) [1–3]. Tumors in the 129-*Ter* strain develop from primordial germ cells (PGCs) during embryonic development [4–7]. A progressive loss of PGCs is observed in *Ter* mice starting at embryonic day (E) 8.5 [8]. Consequently *Ter*/*Ter* mice are sterile at birth. However, in males, some of the PGCs escape death and become transformed to embryonal carcinoma (EC) cells. Clusters of proliferating EC cells are first detected at E15.5 within the embryonic gonads [9,10]. The proliferating EC cells disrupt the normal architecture of the gonads. Soon after birth, the EC cells differentiate into a random mix of differentiated tissues that constitute the tumors.

These effects of *Ter* have been identified to be due to inactivation of the *dead-end* (*Dnd1*) gene [11]. However, tumor development of *Ter* mice occurs in a strain-specific manner such that 94% of 129-*Ter*/*Ter* mice develop testicular tumors. On other or mixed strain backgrounds, loss of functional *Dnd1* results only in PGC depletion and consequently, sterility in *Ter*/*Ter* adults but no significant incidence of germ cell tumor development. The mechanism as to how the loss of *Dnd1* leads to primordial germ cell death or tumor development is unknown.

Dnd1 is expressed in PGCs after E7.25 [12]. Widespread expression of *Dnd1* transcript is also detected in the early embryo after E7.5 [11]. Here, we report that inactivation of *Dnd1* also affects embryonic viability of 129-*Ter* mice.

The mouse *Dnd1* gene encodes two protein isoforms, named DND1-isoform α and DND1-isoform β (or DND1-α and DND1-β, respectively, Fig. 1A). They arise due to alternate splicing of transcripts (Fig. 1A).

We wished to determine if both DND1 isoforms are involved in germ cell tumor development. Using antibodies that detect each DND1 isoform, we found DND1-α

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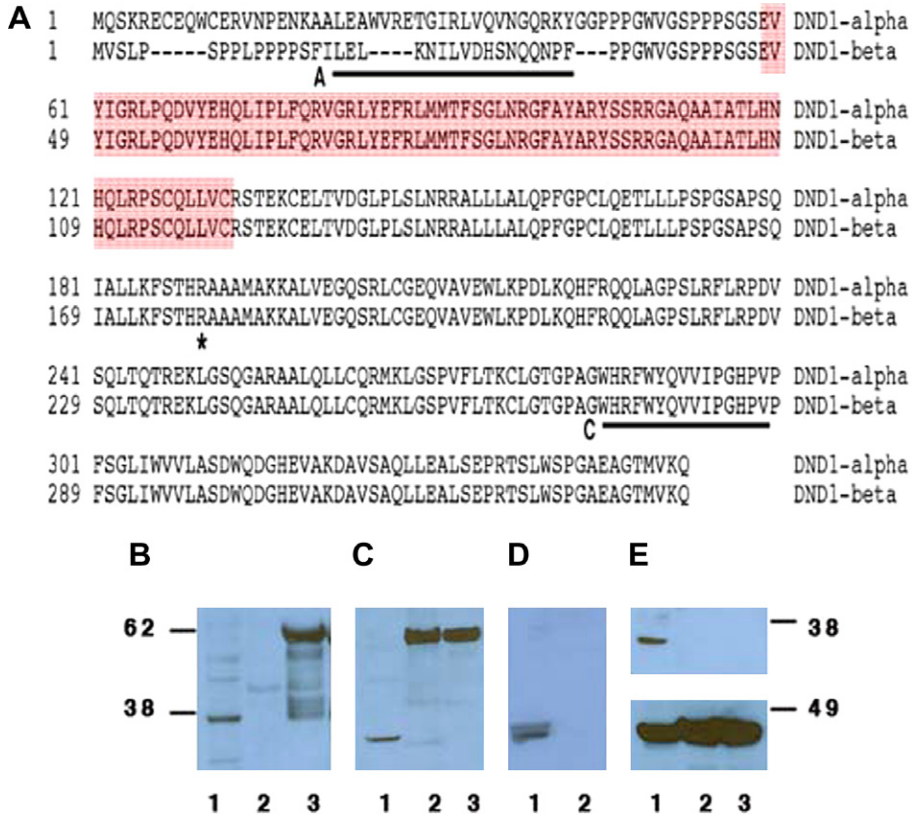

Fig. 1. The mouse DND1-α and DND1-β protein isoforms. (A) Sequence comparison of DND1 isoforms (GenBank: AAQ63636 and AAH34897). A and C (underlined) mark the sequences to generate antibody A and C. The red box indicates the RNA recognition motif. The asterisk (*) marks the amino acid (R) that is mutated to a stop codon in *Ter* mice. (B) Western blotting using antibody A of testes lysate (lane 1); GST–DND1-α (lane 2) and GST–DND1-β (lane 3). (C) Western blotting using antibody C of testes lysate (lane 1); GST–DND1-α (lane 2) and GST–DND1-β (lane 3). (D) Western blotting using both antibody A and C of normal testes (lane 1) and spleen (lane 2). (E) (top panel) Western blotting using antibody C of normal testes (lane 1); germ cell deficient testes from *Ter*/*Ter* (lane 2); testicular tumor from *Ter*/*Ter* (lane 3). Rehybridization of the blot with anti-β-actin antibody (bottom panel).

expression in embryonic cells and tissues whereas DND1-β expression is restricted to germ cells of the adult testis. We therefore pinpoint that loss of DND1-α in *Ter* mice is responsible for PGC loss, germ cell tumor development and partial embryonic lethality.

Materials and methods

Generation of antibodies. Rabbit polyclonal anti-peptide antibody-A (BioSource, MA) was against amino acids 16–33 of DND-β [11] (Ac-CILELKNILVDHSNQQNPF-amide) and antibody-C against amino acids 285–299 of DND1-α or 273–287 of DND1-β (Ac-WHRFWYQV VIPGHPVC-amide). Antibodies were characterized by immunoblottting against tissue lysates known to express DND1, GST–DND1 and by peptide blocking of the antibody prior to hybridization.

Western blotting. This was carried out as described [11] using 25–100 μg protein electrophoresed on 4–12% NuPAGE gradient gels (Amersham-Pharmacia Biotech) before transfer onto membranes.

GST (glutathione S-transferase)–DND1 fusion proteins. *Dnd1* cDNA (AAH34897 and AAQ63636, respectively) were cloned into pGEX-2TK (Amersham Pharmacia Biotech) [11].

Mouse strains and tissue collection. 129-*Ter* (129T1/Sv-+^{p}Tyr$^{c\text{-}ch}$ Ter/+@Na) and B6.129-*Ter* have been described [11]. To collect embryos, females were checked for plugs after timed matings (embryos of newly plugged females are denoted E 0.5). Pregnant females were sacrificed on the 13th and 15th day of pregnancy and dissected to obtain embryos. 4–6 embryos were pooled for protein extraction. E13.5 and E15.5 embryos were dissected to obtain embryonic testes. 4–8 pairs of embryonic testes were pooled for protein extraction.

Cell lines. Sertoli cell lines TM4 (ATCC number CRL-1715), 15P-1 (ATCC number CRL-2618), and MSC1 were cultured as described [13]. EG cells were maintained and passaged on Mitomycin-C arrested primary MEFs (PMEF-CF, Specialty Media, NJ) on media supplemented with LIF (1000 u/mL) and βFGF (1 ng/mL) [14]. EG cells were checked by staining with alkaline phosphatase chromogen (Fast Red tablets, Abcam) (data not shown). G4 ES cell lines were passaged 2 times on feeder-free gelatin-coated plates to remove MEF cells. COS-7 and HeLa were from ATCC.

Fluorescent-tagged DND1. *Dnd1* cDNA (AAH34897 or AAQ63636) were cloned into pEGFP-C1 (BD Biosciences Clontech). The expression plasmids, GPF-DND1-α or -β were transfected separately into cells and visualized 48 h later using *Zeiss* LSM 510 Confocal Microscope.

RT-PCR for Dnd1 transcripts. A 366 bp product from *Dnd1*-α whereas a 700 bp product *Dnd1*-β was amplified using primers Dnd10-F and Dnd10-R (5′-ATGCAGTCCAAACGGGAGTGCGAG-3′ and 5′-CTG GTGGTTGTGCAGCGTAGC-3′, respectively). Primers for hypoxanthine phosphoribosyltransferase (*HPRT*) were: HPRT-F (5′-GTTG AGAGATCATCTCCACC-3′) and HPRT-R (5′-AGCTATGATGAA CCAGGTTA-3′).

Separation of germ cells. Germ cells were separated from cell suspensions prepared from adult testes of five C57BL/6J mice according to general procedures described previously [15]. Three fractions were obtained containing 84% pachytene primary spermatocytes, 90% round spermatids, and a mixture of late spermatids (14%) and cytoplasmic fragments (85%), 90% of which are detached from the late spermatids cells

[16]. Hence, this fraction can be considered as a relatively pure fraction of late spermatid nuclear and cytoplasmic material. The three fractions collected were concentrated and washed with PBS. Cells were lysed and 50 μg used for Western blotting.

Results

Antibodies A and C detect mouse DND1-β and -α isoforms, respectively

We previously characterized, antibody A, a polyclonal antibody against DND1-β (Fig. 1A) [11]. We report here of a second antibody, antibody C (Fig. 1A) designed to detect both DND1-α and DND1-β.

Western blotting indicated that, as expected, antibody A detects only bacterially expressed, recombinant GST–DND1-β as well as a single band from normal testes, DND1-β (Fig. 1B). Antibody C, as expected, detects both recombinant GST–DND1-α and GST–DND1-β (Fig. 1C). However, antibody C detects a single band from normal mouse testes (Fig. 1C) and which is slightly lower in size than the band detected by antibody A (Fig. 1B). The two DND1 isoforms are apparent in testes lysate when the membrane is hybridized with both antibodies A and C simultaneously (Fig. 1D). Thus, although antibody C is able to detect both recombinant GST–DND1-α and -β proteins, it only detects one isoform of DND1 from the mouse testes. Antibody C likely detects the other DND1 isoform, DND1-α, because the band is close to but of different size compared to DND1-β as detected by antibody A.

Based on the amino acid composition, DND1-α is theoretically 39.1 kDa and DND1-β is 37.5 kDa. However, experimentally, electrophoresis of testes lysates on NuPAGE MES (Invitrogen) gels followed by Western blotting with antibody A or C shows a single distinct band near the 38 kDa marker (Fig. 1B and C). Moreover, the band for DND1-β is slightly larger in size compared to DND1-α (comparing lanes 1 of Fig. 1B and C) and thus DND1-β migrates anomalously on the NuPAGE MES (Invitrogen) gels. A likely explanation is that DND1-β is extensively post-translationally modified. This would explain its anomalously larger size compared to DND1-α on gels as well as why DND1-β cannot be detected by antibody C.

Taking together the above observations, we conclude that antibody A detects DND1-β and antibody C detects DND1-α. Both antibodies were tested for specificity for DND1 by previously incubating the antibody with the cognate peptide (peptide blocking) so as to block recognition of recombinant GST–DND1 and testis DND1 (data not shown). In many instances (experiments described below), we hybridized each membrane with antibody A and rehybridized with antibody C. Neither of the antibodies detected DND1 from germ cell deficient testis or tumors from *Ter*/*Ter* mice (Fig. 1E, top panel) or from normal spleen (Fig. 1D) where *Dnd1* is not expressed [11].

DND1-α is expressed in mouse embryos and embryonic gonads

To determine which isoform of DND1 is responsible for germ cell tumor development, we utilized the isoform specificity of the two antibodies, A and C, to determine the expression patterns of DND1 in the primordial gonads of the mouse. Western blotting carried out on lysates from embryonic testes at E13.5 and E15.5 detected DND1-α but not isoform β (Fig. 2A, top and middle panels). Germ cell tumors start developing around E13.5 in the embryonic testes [9,10]. As only DND1-α is detected in embryonic testes at these stages, this implies loss of DND1-α to be the cause of germ cell tumor development in *Ter* mice.

To examine expression of DND1 in germ cells, we used embryonic germ (EG) cells (Fig. 2A) grown in culture. EG cells are derived from PGCs and resemble their founders in many respects [17–20]. We detected low levels of DND1-α in EG cells (Fig. 2A, lane 8, middle panel). To facilitate detection of DND1, the protein concentration of the EG cell lysates was increased to 100 μg. However, because EG cells are grown on a feeder layer of Mitomycin-C arrested primary mouse embryo fibroblasts and lysates were made directly off the cell culture plate, it is likely that we are underestimating DND1 levels in EG cells. There-

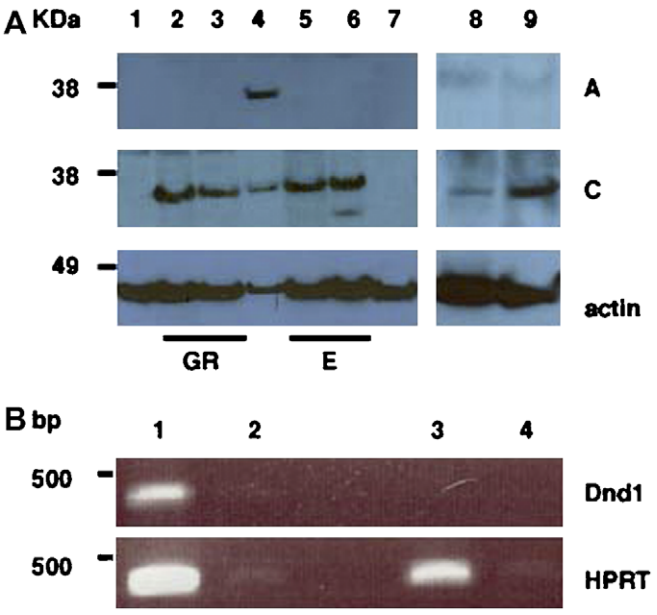

Fig. 2. Expression of DND1-α and DND1-β in embryonic tissues. (A) Lysates from embryonic testes (GR) at E13.5 (lane 2) and E15.5 (lane 3), adult testes (lane 4), E13.5 embryo (lane 5), E15.5 embryo (lane 6), EG (lane 8) and ES cells (lane 9), were electrophoresed before Western blotting with antibody A (top panel); antibody C (middle panel) and anti-β-actin (bottom panel). Negative control: mouse spleen (lane 1) and germ cell tumor lysates from *Ter*/*Ter* mice (lane 7). GR, embryonic testis/genital ridges; E, embryos. (B) (top panel) RT-PCR of total RNA from EG cells (lanes 1 and 2) grown on Mitomycin-C treated MEF cells and Mitomycin-C treated MEF cells alone (lanes 3 and 4). Lanes 2 and 4 are control lanes where no superscript was added during RT. (bottom panel) Control RT-PCR for *HPRT*.

Table 1
Comparing genotypes of progeny derived from 129-*Ter* to B6.129-*Ter*

	Genotype			Total no. examined	χ^2	*P*
	+/+	*Ter*/+	*Ter*/*Ter*			
129-*Ter*	62	98	17	177	23.6	< 0.001
B6.129-*Ter*	39	84	41	164	0.2	0.2

Comparing the number of *Ter*/*Ter* progeny derived from the 129-*Ter* strain to that from a non-129 strain (the B6.129-*Ter* strain). The B6.129-*Ter* strain contains a 5 Mb region from 129, containing the *Ter* mutation, which was made congenic on a C57B1/6J strain background. Crosses were set up using heterozygote (*Ter*/+) parents. Adult progeny of both sexes were genotyped.

fore, we examined *Dnd1* mRNA transcripts by RT-PCR on mRNA from EG cells grown on feeder layers and were able to readily detect expression of *Dnd1*-α transcripts (Fig. 2B). *Dnd1* was not detected in mRNA derived from Mitomycin-C arrested primary mouse embryo fibroblasts (PMEF) alone. Thus, our data indicate that DND1 is expressed in EG cells. Additionally, quantitative single-cell gene expression analysis techniques have detected *Dnd1* transcript in PGCs after E7.25 onwards [12]. Future work will examine protein expression levels of DND1 from purified isolated PGCs of mouse embryos.

Because ES (embryonic stem) cells share many common features including gene expression patterns with EG, PGCs, and EC (embryonal carcinoma) cells [17–22], we examined DND1 expression in one ES cell line (G4 line) and found DND1-α but no DND1-β expression (Fig. 2A, lane 9). The ES cell lysates were extracted from G4 cells passaged twice on gelatin-coated plates to eliminate most of the feeder cells. Thus, DND1-α is also expressed in other types of pluripotent mouse cell lines.

Western blotting on lysates derived from mouse embryos at E13.5 and E15.5 detected expression of DND1-α but not DND1-β (Fig. 2A).

Partial embryonic lethality of Ter/Ter on the 129 mouse strain background

Both *in situ* hybridization [11] and Western blotting, as shown here, indicate that DND1 (DND-α) is expressed in mouse embryos. However, although DND1 is expressed in the early embryo, no obvious effects on development have been reported when DND1 expression is inactivated in the *Ter*/*Ter* mice. We genotyped the progeny of 129-*Ter*/+ intercrosses and found that there was a fourfold reduction in the expected numbers of *Ter*/*Ter* mice ($P < 0.001$) (Table 1). The expected number of 129/Sv-*Ter*/+ progeny was also decreased. We have not observed significant post-natal death of progeny after birth or upon weaning in the 129-*Ter*/+ colony and thus the death of a proportion of the *Ter*/*Ter* mice likely occurs before birth.

We also examined crosses of *Ter*/+ on other strain backgrounds (B6.129-*Ter* strain). In these crosses, normally expected numbers of *Ter*/*Ter* progeny were present (Table 1). Thus, partial embryonic lethality of *Ter*/*Ter* mice occurs only on the 129 strain background.

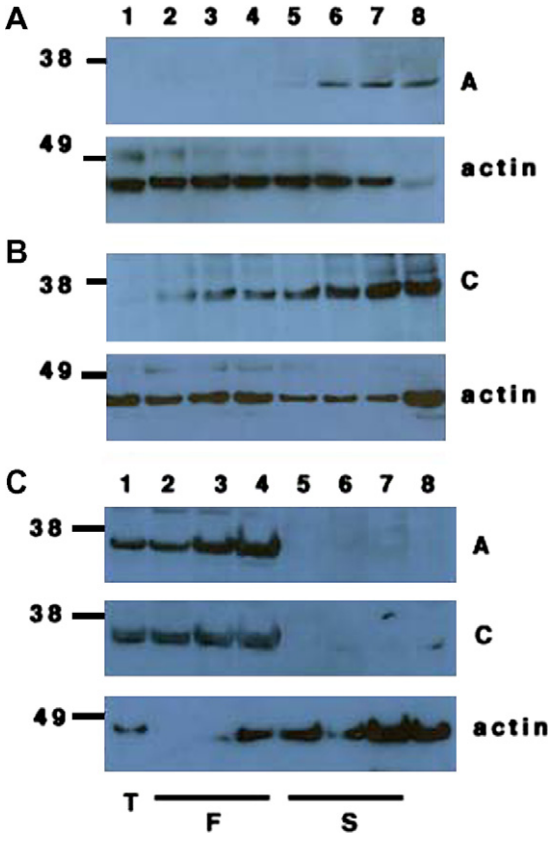

Fig. 3. Expression of DND1-α and DND1-β adult testis. (A) Western botting of lysates from post-natal testes of day 1, 6, 10, 15, 20, 25, 30 and 50 (lanes 1, 2, 3, 4, 5, 6, 7 and 8, respectively) with antibody A (top panel) and anti-β-actin (bottom panel). (B) Western blotting of lysates from post-natal testes of day 1, 6, 10, 15, 20, 25, 30 and 50 (lanes 1, 2, 3, 4, 5, 6, 7 and 8, respectively) with antibody C (top panel) and anti-β-actin (bottom panel). (C) Western blotting of lysates from adult testes (lane 1), fractionated cells from adult testes: pachytene spermatids (lane 2), round spermatids (lane 3) and elongated spermatids (lane 4); Sertoli cell lines, TM4 (lane 5), 15P-1 (lane 6), MSC1 (lane 7), and germ cell deficient *Ter*/*Ter* testis (lane 8) with antibody A (top panel); antibody C (middle panel) and anti-β-actin (bottom panel).

Differential expression of DND1-α and -β in testes of post-natal mice

To determine the pattern of expression of DND1 in post-natal testes, we performed Western blotting of lysates from post-natal (PN) testes collected from mice at intervals from PN1 to PN 50. Results indicated that DND1-α is expressed continuously in PN testes although levels are

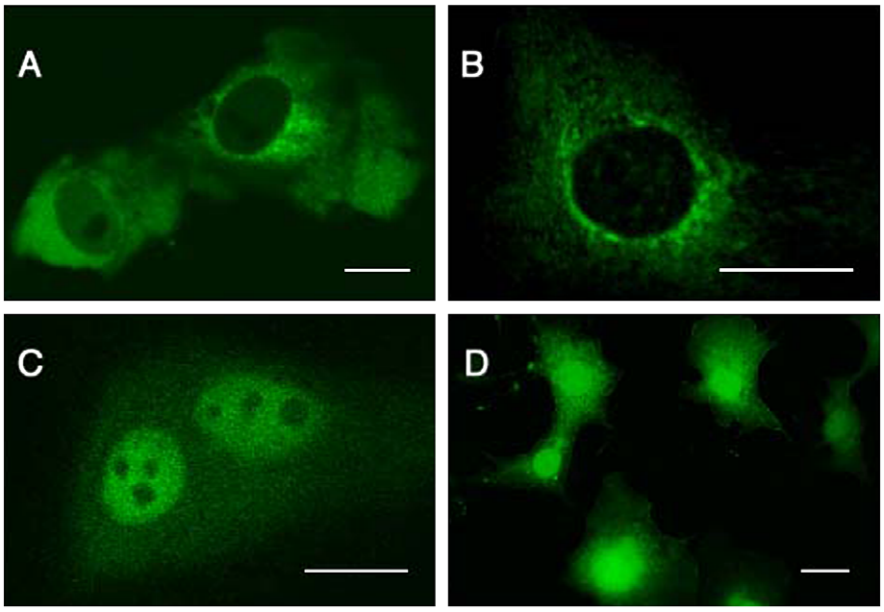

Fig. 4. Mammalian DND1 localizes to the cytoplasm or nucleus in different cell types. Subcellular localization of mouse GFP–DND1-α (A) and GFP–DND1-β (B) in COS-7 cells and He La cells (C). Control transfection of vector encoding GFP only (D) in COS-7 results in GFP expression in both cytoplasm and nucleus. Line indicates 20 μm.

low between post natal day 1 (PN1) and PN6. DND1-β is expressed from PN 20 onwards (Fig. 3A and B).

To test the expression of DND1 in post-meiotic germ cells of adults, we examined elutriated pachytene spermatids, round spermatids and elongated spermatids from adult mouse testes of the C57Bl/6J strain. Higher expression of DND1-β was at the elongated spermatid stage (Fig. 3C). This suggests a role of DND1-β in post-meiotic adult germ cells whereas DND1-α is present and likely required at almost all stages of germ cell development.

Neither DND1-α nor -β was detected by Western blotting of the Sertoli cell lines (Fig. 3C) TM4, 15P-1, and MSC1 [13]. Thus, expression of DND1 is restricted to the germ cells of adult testes.

Subcellular localization of DND1 in mammalian cells

We next examined the subcellular localization of mouse DND1 in mammalian cells. Expression constructs encoding GFP–DND1-α and -β were transfected into COS-7 and HeLa cells prior to examination by confocal microscopy. In COS-7 cells, both DND1-α and -β were localized to the cytoplasm (Fig. 4A and B). However, in HeLa cells, both isoforms of the GFP–DND1 localized to the nucleus (Fig. 4C and data not shown). DND1 shows homology with ACF (apobec-1 complementation factor). ACF possesses a canonical SV40-like nuclear localization signal as well as a novel 41-residue nuclear localization signal (ANS) [23] that allows it to actively shuttle between the cytoplasm and nucleus. However, DND1 lacks the ANS and shows no significant homology to the SV40-like nuclear localization signal. Thus, the mechanism as to how mouse GFP–DND1 migrates to the nucleus of some cell types is at present not understood.

Discussion

We had previously identified that inactivation of *Dnd1* causes PGC loss and germ cell tumor development in the *Ter* mouse strain. Two isoforms of *Dnd1* are detected in adult testes [11]. We initially generated an antibody against DND1-β isoform [11]. Here, we report generation of antibody against DND1-α.

Although antibody C should theoretically recognize both isoforms of DND1, it was only able to recognize DND1-α from the tissue lysates. Moreover, DND1-β appears to be larger in size than expected. These two observations lead us to conclude that DND1-β is likely post-translationally modified, thus explaining its anomalous larger size and inability to be detected by antibody C. Future work will focus on the experimental identification of the post-translational modifications of DND1-β that blocks its recognition by antibody C.

Mouse embryonic gonads at E13.5 and E15.5 express DND1-α. As germ cell tumors in mice develop at around E13.5, this observation implicates loss of DND1-α in *Ter* mice to be the cause of tumor development. We examined EG cells in culture [18] for DND1 expression and detected low levels of DND1-α but no DND1-β expression. *Dnd1* transcripts were detected by RT-PCR of EG cell RNA and other investigators have reported presence of *Dnd1* transcripts in PGCs at E7.25 [12]. PGC death starts at E8.5 in *Ter* mice [8] and PGC numbers start to decline gradually from this stage onwards. Taking these observa-

tions together implicates loss of DND1-α as the cause of PGC death at E8.5 onwards in the *Ter* mice.

We show that DND1-α is expressed in embryos at E13.5 and E15.5 thus confirming the previous *in situ* hybridization data on whole embryos [11]. However, the developmental consequences of lack of *Dnd1* in embryos have not been examined rigorously. When we examined the 129-*Ter* colony, we found a deficit of *Ter*/*Ter* progeny due to partial embryonic lethality of *Ter*/*Ter* mice. On other strain backgrounds, lethality of *Ter*/*Ter* is not observed suggesting that modifiers or compensatory factors that rescue embryonic lethality are present in these strains. The embryonic lethality of 129-*Ter*/*Ter* mice is likely due the role of *Dnd1* in critical organ systems in the developing embryo. For example, *in situ* hybridization indicated *Dnd1* expression in the ventral neuroectoderm at E8.5 and in the head mesenchyme and first branchial arch at E9.5. The stage at which this partial embryonic lethality occurs remains to be determined.

Our studies show that DND1-α is expressed continuously in testes of post-natal (PN) mice. Although DND1 has been shown to be required for PGC viability in zebrafish, xenopus and mouse, our data suggest that DND1-α could be required for viability of post-natal and adult germ cells of the mouse as well. DND1-β is expressed from PN 20 onwards in meiotic and in post-meiotic adult germ cells of the testes with highest expression at the elongated spermatid stage.

We found that mouse DND1 localizes to the cytoplasm or the nucleus depending on the mammalian cell type. The sub-cellular localization of mouse DND1 may be a reflection of the rate of shuttling of GFP–DND1 between the two cellular compartments in different cell types or it may be an inherent consequence of the cell type. Future work will examine the subcellular distribution of DND1 in different cell types of the mouse embryo. This may provide clues about DND1 function in different tissues and cell types during development.

In summary, isoform-specific antibodies show that DND1-α is expressed in the early embryo and gonads. Our data implicate loss of DND1-α to cause PGC death as well as testicular tumor development and partial embryonic lethality in the 129-*Ter* strain.

Acknowledgments

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