The RNA-binding protein DND1 acts sequentially as a negative regulator of pluripotency and a positive regulator of epigenetic modifiers required for germ cell reprogramming

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ABSTRACT

The adult spermatogonial stem cell population arises from pluripotent primordial germ cells (PGCs) that enter the fetal testis around embryonic day (E) 6.75. They proliferate, migrate through the gut mesentery and arrive in the allantois in mouse embryos at embryonic day (E) 6.75. They then enter prolonged cell cycle arrest (G1/G0), during which they transition to pro-spermatogonia. In mice homozygous for the Ter mutation in the RNA-binding protein Dnd1 (Dnd1Ter/Ter), many male germ cells (MGCs) fail to enter G1/G0 and instead form teratomas: tumors containing many embryonic cell types. To investigate the origin of these tumors, we sequenced the MGC transcriptome in Dnd1Ter/Ter mutants at E12.5, E13.5 and E14.5, immediately prior to teratoma formation, and correlated this information with DO-RIP-Seq-identified DND1 direct targets. Consistent with previous results, we found DND1 controls downregulation of many genes associated with pluripotency and active cell cycle, including mTor, Hippo and Bmp/Nodal signaling pathway elements. However, DND1 targets also include genes associated with male differentiation, including a large group of chromatin regulators activated in wild-type but not mutant MGCs during the E13.5 and E14.5 transition. Results suggest multiple DND1 functions and link DND1 to initiation of epigenetic modifications in MGCs.

KEY WORDS: DO-RIP-Seq, Dnd1, Germ cells, RNAseq, Teratoma

INTRODUCTION

Primordial germ cells (PGCs) are specified at the base of the allantois in mouse embryos at embryonic day (E) 6.75. They proliferate, migrate through the gut mesentery and arrive in the gonad between E10.0 and E11.0, expressing stem cell markers that include SOX2 (SRY-box 2), NANOG (Nanog homeobox) and OCT4 (also known as Pou5F1, Pou domain, class 5, transcription factor 1) (Matsui et al., 1992; McLaren, 1995; Yeom et al., 1996; Pesce and Schöler, 2000; Yamaguchi et al., 2005). Prior to E12.5, germ line stem cells (EGCs) can be derived readily from the germ cell population (Matsui et al., 1992), and teratomas, tumors in which all embryonic cell types are represented, spontaneously arise from EGCs in males with a frequency of 1-10% in some strains (Steens and Little, 1954; Bustamante-Marin et al., 2013; Dawson et al., 2018). However, between E12.5 and E15.5, the efficiency of both EGC derivation and teratoma induction declines, reflecting epigenetic and gene expression changes that lead to suppression of the underlying pluripotent state of germ cells (Gu et al., 2018; Ng et al., 2013). These changes are coincident with the entry of male germ cells into G1/G0 cell cycle arrest and their fate transition to pro-spermatogonia (Fig. 1A) (Stevens, 1966; Western et al., 2011, 2010).

A mutation, called Ter, that arose spontaneously during routine breeding of 129/SvJ mice, caused a severe reduction in germ cell numbers soon after their specification and acted as a strong modifier that increased the incidence of spontaneous testicular teratomas to >90% in 129/SvJ mutants (Fig. 1A) (Stevens, 1973). In 2005, Ter was mapped to a point mutation that introduced a premature stop codon in the RNA-binding protein (RBP) dead end 1, Dnd1 (Dnd1Ter) (Fig. 1B) (Youngren et al., 2005). Understanding how DND1 is involved in stabilization of germ cell fate will provide important insights into how this crucial process is controlled in germ cells and, by analogy, in other stem cell populations from which tumors arise.

The function of DND1 has been studied by knockouts in zebrafish (Cross-Thebing et al., 2017; Weidinger et al., 2003) and mouse (Li et al., 2018; Zechel et al., 2013), and by a conditional deletion at E13.5 in mouse (Suzuki et al., 2016). Loss-of-function alleles of Dnd1 led to lethality or complete germ cell loss. However, the increase in teratoma incidence is specific to the presence of the Dnd1Ter mutation on the 129 genetic background. With the goal of understanding the role of DND1 in the transition from PGC to teratoma, we focused our efforts on characterizing the transcriptome in germ cells from Dnd1Ter/129SvT2/SvEMSJ (129T2) mutant embryos immediately prior to the time when teratomas form (Cook et al., 2009, 2011; Heaney et al., 2012) and cross-referencing this information with direct binding targets of DND1.

Previous studies have implicated DND1 in both positive and negative regulatory roles. The molecular role of DND1 was first investigated in a human tumor cell line where the protein was shown to bind to the mRNAs of Cdkn1b (cyclin-dependent kinase inhibitor 1B, a negative regulator of the cell cycle) and Lats2 (large tumor suppressor 2, a tumor suppressor and negative regulator of p53) to protect these transcripts from miRNA-mediated translational repression (Kedde et al., 2007), perhaps through interaction with APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) (Bhattacharya et al., 2008). Other studies identified targets protected by DND1 in NIH3T3 or HEK293 cells, including Cdkn1b and Ezh2 (enhancer of zeste homolog 2), a mediator of H3K27me3 repression (Cook et al., 2011; Gu et al., 2018). Recent work in Xenopus showed that DND1 promotes translation of nanos1 (C2HC type zinc finger 1) by relieving the
inhibitory function of the eukaryotic initiation factor 3f (eIF3f), a repressive component of the preinitiation complex (Aguero et al., 2017), and that the second RNA recognition motif (RRM2) exhibits ATPase activity required for this function (Aguero et al., 2018). In contrast, two labs showed that DND1 acts as a negative regulator of mRNAs by recruiting targets to the CCR4-NOT deadenylase complex for degradation (Suzuki et al., 2016; Yamaji et al., 2017). Suzuki et al., (2016) showed that DND1 acts as an essential partner of NANOS2 (C2HC type zinc finger 2, another male-specific RBP) to form P bodies, and to select and recruit mRNA for degradation. In these studies, conditional deletion of Dnd1 at E13.5 led to upregulation of proliferation, meiosis markers and caspase 3, and the downregulation of several NANOS2 targets associated with male differentiation, similar to Nanos2 mutants (Suzuki et al., 2016). CRISPR-Cas9 analysis identified four missense mutations in the first RNA recognition motif (RRM1) that decrease the interactions of DND1 with NANOS, but not with the CNOT complex. All of these mutations led to a failure of germ cell development by E12.5 (Li et al., 2018). The NMR spectroscopy-based solution structure of DND1 bound to a target RNA revealed that RRM1 acts as the main binding platform with the cooperation of RRM2 to stabilize binding to targets, and a role for the double-
stranded RNA-binding domain in the recruitment of factors that mediate target repression (Duszczyk et al., 2019 preprint).

Most studies have relied on a candidate approach to identify targets of DND1, which included pluripotency factors and cell cycle genes (Cook et al., 2011; Gu et al., 2018; Kedde et al., 2007; Zhu et al., 2011). More recently, Yamaji and co-workers performed photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) assays in HEK293 cells, and investigated the expression of identified targets in induced primordial germ-like cells (PGLCS) and germine stem cells derived from adult spermatogonia. Consistent with Suzuki’s results, Yamaji et al. showed that DND1 acts with CCR4-NOT to destabilize and repress RNAs associated with apoptosis, inflammation and signaling pathways, including TGFβ (transforming growth factor β), Wnt (wingless-type MMTV integration site family) and PI3K-AKT (serine-threonine protein kinase). Extrapolating results from these in vitro cell types, Yamaji et al. suggested that activation of pluripotency genes, as well as TGFβ and inflammatory signaling genes, contribute to the formation of teratomas in gonadal germ cells (Yamaji et al., 2017).

We sequenced the transcriptome of male germ cells in Dnd1Ter/Ter mutants at E12.5, E13.5 and E14.5, immediately prior to the formation of teratomas, and correlated this information with direct targets of DND1. We identified direct transcript targets of DND1 using an independent method, digestion optimized-RIP-seq (DO-RIP-seq), after expressing tagged DND1 proteins in HEK293 cells. We cross-referenced our DO-RIP-seq targets with the PAR-CLIP results from Yamaji et al., and compared the transcript levels of nontargets and targets common to both RNA-immunoprecipitation assays in isolated germ cells from 129T2-Dnd1Ter/Ter and Dnd1+/+ littermates at E12.5, E13.5 and E14.5. Consistent with our previous results and with the molecular models proposed by Suzuki and Yamaji, between E12.5 and E13.5 DND1 controls the downregulation of many genes associated with pluripotency and the cell cycle, including elements of the both the Hippo and Bmp/Nodal signaling pathways. However, we found that DND1 targets include many genes associated with male differentiation, including a large group of chromatin regulators normally activated during the second transition between E13.5 and E14.5. These results suggest biphasic functions of DND1, and link DND1 to the initiation of epigenetic modifications in male germ cells.

RESULTS

Dnd1Ter/Ter mutant germ cells are delayed in the germ cell transcriptional profile relative to wild type at E12.5

To investigate the changes in Dnd1Ter/Ter mutant germ cells relative to wild type, 129T2-Dnd1Ter/+; Oct4-EGFP males and females were intercrossed, and ∼600 germ cells were isolated by FACS from four independent homozygous and wild-type fetal gonads at each of 3 stages, E12.5, E13.5 and E14.5, after germ cells have populated the gonads and before teratoma detection (Fig. 1A; Cook et al., 2011; Dawson et al., 2018; Gu et al., 2018). Sequencing was performed, reads were mapped to the genome, and a reads per kilobase transcript per million (RPKM) value was calculated for each transcript. RPKM values used in our analysis are provided with the capability to generate an expression graph for any gene, as a user-friendly resource for the community (Table S1).

The Dnd1 transcript was detected at similar levels in wild-type and homozygous mutant germ cells at E12.5 (Fig. 1B), but showed a rapid decline at later stages (Fig. S7A). The Ter mutation, which is predicted to lead to a premature stop codon (Youngren et al., 2005), is evident in the third exon of Dnd1 and efficiently distinguishes wild-type and mutant samples (Fig. 1B, blue arrow). The 129T2 variant of a SNP that differs between B6 and 129 was detected in the 3’UTR of all samples confirming the 129T2 strain background (red arrow).

Dnd1 is expressed in both XX and XY germ cells from early migratory stages (Youngren et al., 2005). In Dnd1Ter/Ter mutants, many germ cells are lost before arrival in the gonad (Cook et al., 2009; Noguchi and Noguchi, 1985). Given that loss of RBPs (including DND1) in germ cells of other species results in transdifferentiation to somatic fate (Ciosk et al., 2006; Gross-Thebing et al., 2017; Updike et al., 2014), we investigated the possibility that Dnd1Ter/Ter mutant germ cells were significantly altered by the time they populated the mouse gonad, and did not retain their germ cell identity. The top two principal components account for 43% and 25% of the observed variation in the top 500 most variable protein-coding genes. Based on the variability captured by principal component (PC) 1, it appears that E13.5 mutant germ cells cluster more closely with E12.5 wild-type germ cells whereas E12.5 mutant germ cells are delayed. Furthermore, PC2 appears to stem mainly from expression variation in E14.5 Dnd1Ter/Ter germ cells, suggesting that the transcription profile in these mutant germ cells continues to diverge from a normal trajectory (Fig. 1C). To investigate these differences further, we compared expression in Dnd1Ter/Ter mutant germ cells with a list of genes specific to germ cells (Fig. 1D) and a list of genes that distinguish male germ cells (Fig. S2A) at E12.5 and E13.5. These lists include genes that characterize germ cells by their specific expression in this lineage, and those that characterize germ cells by their specific repression (e.g. somatic genes) (Jameson et al., 2012). Based on heat-map analysis, Dnd1Ter/Ter mutant germ cells show an overall pattern similar to wild-type male germ cells for both up- and downregulated genes at E12.5 and E13.5. For example, both Ddx4 (dead box helicase 4) (Vasa) and Dazl (deleted in azoosperma like) show a delay in activation, similar levels at E13.5 and a downregulation at E14.5, whereas Stella (Dppa3 (developmental pluripotency associated protein 3), a germ cell specific gene] and Prdm1 (PR/SET domain 1; Blimp1) show no significant differences from wild type at any stage (Fig. 1E). We performed GO analysis and ingenuity pathway analysis (IPA) on this data, but neither approach provided further insight into the mutant phenotype beyond that presented in other contexts within this manuscript. Overall (Table S2), these data indicate that, although Dnd1Ter/Ter germ cells have a delayed gonadal differentiation program, they have a transcriptome similar to wild-type germ cells soon after they arrive in the gonad.

Neither the female germ cell program nor other embryonic pathways are activated in XY Dnd1Ter/Ter germ cells

Because Dnd1 expression becomes specific to the male pathway after germ cells enter the gonad (Youngren et al., 2005), we next investigated whether there was evidence for a shift to the female germ cell-specific program in Dnd1Ter/Ter male germ cells by comparing expression in mutants to a list of genes specific to female germ cells (Jameson et al., 2012). A heat map comparison showed little evidence for ectopic activation of female pathway genes (Fig. S2B). Based on a cumulative distribution plot, few female-specific genes were significantly altered in mutants at E13.5 (Fig. S2C). In contrast to their pattern in wild-type female germ cells, genes that are associated with entry into meiosis [e.g. Stra8 (stimulated by retinoic acid 8) and Sycp3 (synaptoplasm complex protein 3)] were not upregulated in Dnd1Ter/Ter mutant XY germ
Transposable elements and piRNA pathways were not strongly affected by E14.5, despite the failure to activate their repressors

Several genes involved in repression of transposable elements, including Piwld (piwi like RNA-mediated gene silencing 4), Tdrd5 (tudor domain containing 5), Morc1 (MORC family CW type zinc finger 1) and Mael were either not activated or were expressed at much lower levels in mutants (Fig. 2F). Proteins encoded by these genes normally silence LINE and other transposable elements. Our transcriptome data was based on oligo-dT priming; however, some transposable elements contain polyadenylation sites (Lee et al., 2008). We interrogated the raw transcriptome data at E14.5 for activation of transposable/repetitive elements using RepEnrich, a program optimized to detect differential enrichment between two RNA-seq datasets (Criscone et al., 2014). Very few transposable element reads showed a significant difference between wild-type and mutant germ cells (FDR<0.05) (Fig. 2G). This trend of similarity between wild-type and mutant germ cells was also observed when we interrogated repetitive elements and transposable elements more generally (Fig. S4A,B).

DND1 was previously reported to protect transcripts from miR470- (in zebrafish), and mir-1-, miR-206- and miR-221- (in human cell lines) mediated degradation (Kedde et al., 2007). Therefore, we predicted that targets of a mouse ortholog of these miRNAs, such as miR302 (the ortholog of zebrafish miR470), would be downregulated in the absence of DND1 protection. However, only Lats2 (large tumor suppressor kinase 2) showed this behavior; other targets of miR302 were elevated in Dnd1Tert/Tert germ cells relative to wild type (Fig. S5A). We used IPA to infer activation of individual miRNAs on a global basis. IPA compares the behavior of sets of miRNA targets among samples, calculates a P-value based on this overlap and returns a Z-score reflecting the probability that a particular miRNA pathway is activated or repressed based on the expression of target genes. Based on IPA analysis, only one miRNA (mir223) cleared the activation cut-off of 2 between E13.4 and E14.5 in mutant cells (Fig. S5B). However, investigation of the expression of predicted targets of mir223 revealed no consistent pattern (data not shown).

Identification of DND1 direct targets

We identified hundreds of genes that were up- and downregulated between E12.5 and E14.5 in DND1 mutant PGCs (Fig. 2A). DND1 has been shown to both promote degradation of target transcripts (Suzuki et al., 2014; Yamaji et al., 2017) and to stabilize other target transcripts by competing for miRNA-binding sites (Kedde et al., 2007; Zhu et al., 2011). We were interested in whether genes in both up- and downregulated categories were direct targets of DND1.

Dnd1 shares sequence similarity with genes encoding five other RNA-binding proteins: Rbm46 (RNA-binding motif protein 46), A1cf (APOBEC complementation factor), Rbm47, Syncrip (synaptotagmin-binding cytoplasmic RNA interacting protein) and Hnrnpr (heterogeneous nuclear ribonucleoprotein R). All members of this subfamily contain three RNA recognition motifs (RRMs), except DND1, which contains two RRMs. Additionally, three members of the family, including DND1, contain a C-terminal double-stranded RNA-binding domain (DSR). (Fig. 3A).

The mutation in Dnd1Tert is predicted to lead to a premature STOP codon that disrupts the second RNA-recognition domain (arrow) and eliminates the DSR-binding domain. Whether or not the mutant allele functions as a dominant negative remains unclear. To identify binding sites of DND1, we performed DO-RIP-seq (Nicholson...
et al., 2017) in HEK293 cells, using a tagged mouse DND1 and a tagged DND1-Ter allele. We introduced expression plasmids for both proteins and performed western blot analysis to show that both FLAG-DND1 and FLAG-DND1-Ter proteins were produced in the cells, although FLAG-DND1-Ter was detected at significantly lower levels (Fig. S7B). In accordance with this finding, after pull-down, the autoradiograph of the RNA bound to DND1-Ter was indistinguishable from the negative control, suggesting that little, if any, RNA immunoprecipitated with DND1-Ter (Fig. S7C,D). A single DO-RIP-seq against DND1-Ter was marginally successful with RNA enrichment that was distinct from the negative control. Enriched RNAs in the DND1-Ter pull-down overlapped with 75% of enriched RNAs from DO-RIP-seq against DND1. As efforts to repeat this assay were not successful, there was not sufficient evidence to decisively support or refute the inability of DND1-Ter to efficiently bind transcripts affecting the regulation of these targets. Conclusive functional evidence on DND1-Ter will require further studies employing alternative methods.

Fig. 2. Dnd1Ter/Ter mutant germ cells show delayed suppression of pluripotency, and a failure to activate the male differentiation program. (A) Scatter plots illustrating dynamic changes in expression of germ cell-specific genes (data from Jameson et al., 2012) between mutant (y-axis) and wild type (x-axis) during the transitions between E12.5 and E13.5, and E13.5 and E14.5. Between E12.5 and E13.5, more genes (151) show a higher log2 fold change (L2FC) in mutants than in wild type, whereas only 58 genes lag behind. In the transition between E13.5 and E14.5, the reverse is true: more genes lag behind wild-type expression (266). Of the 43 genes that show a higher L2FC in this interval, many belong to cell cycle or other signaling pathways. Points with L2FC between mutant and wild type: >0.5 in purple; <0.5 in blue. (B) Examples of four genes associated with pluripotency (Dppa5a, L1tf, Nanog and Tdp2c) that show a delay in downregulation in Dnd1Ter/Ter mutant germ cells. (C) Examples of four genes associated with initiation of the male pathway (Dppa4, Nanos2, Ret and Nanos3) that fail to be activated. Mean data are plotted with individual data points shown. (D) Consistent with this, based on GSEA analysis, targets of Dppa4 are not enriched in mutant germ cells during the E13.5-E14.5 transition. (E) Some transcriptional changes in Nanos2 mutants are discordant with changes in Dnd1Ter/Ter mutants. Of genes upregulated in Nanos2 mutants, 11 behave the same (blue) and 40 (red) are downregulated in Dnd1Ter/Ter. Of genes downregulated in Nanos2 mutants, 51 are also downregulated in Dnd1Ter/Ter (blue) and 35 are upregulated (red) (Saba et al., 2014). Only genes with significant L2FC are shown (P<0.05). See also Table S5. (F) Examples of genes associated with suppression of transposable elements and the piRNA pathway are activated in wild type but not mutants. (G) Although many silencers of repetitive elements were downregulated, RepEnrich analysis showed that transposable elements did not exhibit significant aberrant activity at E14.5. Transposable element distribution organized by class. Outer ring: class distribution of elements represented in reads at E14.5 (yellow, LTRs; green, SINEs; red, DNA transposons; blue, LINEs). Inner circle divides specific classes into reads with significant differences between mutant and wild type (bold colors: FDR<0.05; pale colors: FDR>0.05).
In contrast, DO-RIP-seq for the wild-type DND1 allele was very successful. The most-enriched motif in DND1-binding sites was DAUBAW (D=A, G or U, B=C, G or U and W=A or U), a motif that is very similar to the known RNA motif of RBM47, its closest relative (Fig. 3B). The RBM47 motif defined by RNA-Compete (Ray et al., 2013) is GAUSAW (S=G or C and W=A or U), both the DND1 and RBM47 motifs contain AU in the second and third position, and AW in the fifth and sixth, but differ in the first and fourth position where the recognition sequence is more degenerate.

Although Rbm47 expression declines sharply between E12.5 and E14.5, Rbm46, Rbm47, Syncrip and Hnrnpr are all expressed in germ cells in the gonad, and could partially compensate for loss of DND1 (Fig. S6).

Compared with their proportional representation across the whole transcriptome, DND1-binding sites were strongly over-represented in 3′ untranslated regions (UTRs) and to a lesser extent in the coding sequence (CDS) (Fig. 3C). Targets identified by DO-RIP-seq largely agree with PAR-CLIP-identified DND1 targets from Yamaji et al., with 2953 mRNAs shared in common (Yamaji et al., 2017) (Fig. 3D, Table S5). This represents a significant overlap (P<0.001) based on 1000 iterations of randomly shuffled binding sites matched for size and restricted to expressed 3′ UTRs. We suspect that the relatively minor differences between the studies are the result of differences in experimental approaches, and targets that are unique to one study are likely just subthreshold in the other.

**Direct targets of DND1 are both up- and downregulated in Dnd1TER/TER mutants**

Because RIP assays were not conducted in germ cells due to the absence of appropriate antibodies or transgenic tags, we first identified the set of conserved targets expressed in germ cells (1347 out of 2953 targets). Next, we investigated the relationship between number of DND1-binding sites in the target transcript (1 to 9+ binding sites) and its expression in wild-type and 129T2 Dnd1TER/TER mutant germ cells between E12.5 and E13.5, and E13.5 and E14.5. Cumulative distribution function (CDF) plots were generated.
comparing RNA abundance changes for transcripts classified by number of DND1-binding sites (non-targets, 1 site, 2 sites, etc.). Changes in RNA abundance were evaluated in this manner for four conditions: E12.5-E13.5 and E13.5-E14.5 in both mutant and wild-type germ cells. In wild-type germ cells, the presence of more DND1-binding sites in a target transcript was correlated with a higher likelihood of downregulation relative to transcripts with few or no target sites during the transition between E12.5 and E13.5, whereas the opposite pattern was observed between E13.5 and E14.5 – target transcripts with more DND1-binding sites were more likely to be upregulated relative to non-targets. During this later transition, DND1 targets tend to change faster than the rest of the transcriptome (Fig. 3E). In 129T2-Dnd1Ter/Ter mutants, DND1 targets behave more like non-targets, reflecting the effect of the Ter mutation on DND1 function.

Overall, among targets of DND1, cell cycle genes and chromatin regulators were over-represented (Fig. 4A; Table S5). Overlapping binding sites from all three RIP assays were present in the 3′UTR of the cell cycle gene Ccne1, and throughout the CDS and 3′UTR of the chromatin regulator Kat7 (lysine acetyltransferase 7) (Fig. 4B). In addition to Ccne1, Rheb (Ras homolog enriched in brain) and Rhob (Ras homolog family member B), two GTP-binding proteins associated with active cell cycle were targets of DND1, and were elevated in the mutant transcriptome along with Trp53 (transformation related protein 53), which was not expressed in HEK293 cells (Fig. 4C). Kat7 and other chromatin regulators, including Setdb1 (set domain bifurcated 1), Kdm5a (lysine demethylase 5a) and Rbbp5 (RB-binding protein 5, histone lysine methyltransferase complex subunit) mapped as targets, and all lagged behind wild-type levels (Fig. 4D), as did many other chromatin regulators not expressed in HEK293 cells (Fig. S8). Many genes associated with signaling pathways were identified as targets of DND1, and were significantly changed in mutants (Fig. 5A). A large group of genes associated with the BMP signaling pathway were elevated in mutant germ cells (Fig. 5B), as also reported by Yamaji (Yamaji et al., 2017). We identified elements of other signaling pathways elevated in mutants that map as targets of DND1, including Ywhah (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta), Ywhag (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma), Csnk1e (casein kinase 1 epsilon), Yap1 (yes-associated protein 1) and Prkci (protein kinase Cι) in the Hippo pathway (Fig. 5C). In contrast, transcripts for two central genes in the mTOR pathway that mapped as direct targets of DND1, Rictor (rapamycin-
Insensitive companion of mTOR (mammalian target of rapamycin), were downregulated in mutants (Fig. 5D).

Ingenuity pathway analysis identified apoptotic and cell cycle pathways as strongly affected in mutants.

We used IPA to identify curated pathways most strongly affected in the mutant transcriptome. This analysis resulted in the identification of cellular growth and proliferation (482 genes) and cell death and survival (433 genes) as the most affected pathways (Fig. S9A-C). These results are consistent with previous findings that many Dnd1Ter/Ter germ cells do not enter cell cycle arrest, but are eliminated through apoptotic pathways (Cook et al., 2009; Dawson et al., 2018; Yamaji et al., 2017).

DISCUSSION

Germ cells are an important model for investigating how stem cells embark on a highly specific differentiation pathway while restricting their potential for spontaneous differentiation or tumorigenesis. Although most germ cells appear to strictly control this potential and to successfully navigate this transition, a small subset of 129T2 Dnd1Ter/Ter mutant germ cells fail this test and spontaneously differentiate into teratomas – and in so doing provide a tool for investigating the genetic regulation of germ cell differentiation.

DND1 likely performs several roles in post-transcriptional regulation. As previously shown (Ng et al., 2013), E13.5 is a clear transition point between the embryonic and fetal programs: functions of DND1 may segregate into early exit from the pluripotency program, involving the degradation of transcripts for many pluripotency genes, and subsequent activation of transcripts associated with the male pro-spermatogonial pathway. Although we could not detect pluripotency targets in HEK293 cells, Matin and co-workers previously reported Oct4, Nanog, Sox2, Lin28 (LIN28 family RNA-binding protein), Bax (BCL2 associated X apoptosis regulator), and Bmp4 (Bone morphogenetic protein 4) as targets of DND1 (Ng et al., 2013).
mediated knockout of regulators was not reported in the original of mutant germ cells to initiate differentiation to multiple cell types (Saba et al., 2014). However, heterozygosity for a CRISPR/Cas9-mediated knockout of Nanos2 on the 129/SvJ background led to a decrease in DNMT3L expression, a defect in re-methylation of the male germ cell genome, elevated levels of Line-1 and an increase in teratoma incidence (Dawson et al., 2018). Although links between epigenetic regulation and RBPs have been well described in C. elegans (Strome and Updike, 2015), they are poorly understood in mammalian systems.

Many genes associated with the cell cycle are targets of DND1 and are dysregulated in mutants (this study; Cook et al., 2011; Yamaji et al., 2017). Several signaling pathways, including Kit, Fgf, Nodal and Hippo are also elevated and could be responsible for driving ongoing proliferation. A failure to exit active cell cycle and enter G0 arrest is a characteristic of mutant germ cells and teratoma formation, but whether this failure is causal remains unclear. The association of FOXH1 with the YAP/SMAD complex is believed to drive the switch from pluripotency to differentiation of mesendoderm (Slagle et al., 2011), but further studies would be required to determine whether this finding has relevance for the differentiation of cell types in teratomas.

Based on the failure to activate many repressors of transposable elements, such as Piwi4, Morc1, Mael and Tdrd5, we anticipated a strong activation of LINEs and other transposons. However, at the latest stage of our analysis (E14.5), we did not detect significant changes in genes in this category. We were also surprised by the absence of significant changes in mirRNA regulatory signatures, given the evidence that DND1 protects transcripts from miRNA-mediated degradation (Kedde et al., 2007). One possibility is that the protective activity of DND1 occurs at later stages of germ cell development. However, we are cautious about drawing strong conclusions about these data, as Z-scores used in the IPA may not be based on important targets specific to germ cells. Similarly, despite the absence of many epigenetic regulators that normally characterize the male germ cell transcriptome at these stages, we found no evidence of lineage infidelity. One possibility is that E14.5 (the latest stage of this analysis) is too early to detect these changes at the population level. There is significant variability in the response of individual germ cells to the Dnd1Ter mutation. Dnd1Ter mutant germ cells transform and form teratomas at a much higher rate than wild-type germ cells. However, even though the ‘per mouse’ teratoma rate is high, the ‘per germ cell’ transformation rate is still quite low. Most Dnd1Ter germ cells undergo apoptosis, as evidenced by the strong footprint of cell death and cell cycle disruption at the population level.

It would be very instructive to know the steps involved in the transition from a germ cell to a teratoma. Dawson and colleagues have recently suggested that this involves a transition through a primed pluripotent EC cell state (Dawson et al., 2018). The fact that only a few germ cells form teratomas in a Dnd1Ter testis, whereas other germ cells nearby either die or continue to express normal germ cell markers (Cook et al., 2011; Dawson et al., 2018; Gu et al., 2018), suggests that the trigger must be a threshold event that is influenced by cell-autonomous fluctuations of factors in germ cells and/or by the microenvironment in the testis of some strains. Teratoma formation likely competes with apoptotic pathways that are strongly activated at all stages of our mutant transcriptome analysis. In this study, we have identified many targets of DND1 and captured their transcriptional changes in Dnd1Ter germ cells. However, the specific transcriptional events in the rare cells undergoing the transition to teratoma may be obscured by the transcriptomes of the large number of cells that are not. We plan to use a single cell analysis in future experiments to resolve this problem.

Finally, the role played by the Dnd1Ter allele in the teratoma phenotype remains unclear. Recent work has implicated the RRM2 and DSR domains, both disrupted by the Ter mutation, as important mediators of DND1 activity (Aguero et al., 2018; Duszczyk et al., 2019preprint). We show that RNA from the Dnd1Ter allele was produced at near normal levels when germ cells first arrive in the gonad (Fig. 1), but declined to very low levels at E13.5 and E14.5 (Fig. S10); thus, if the DND1-Ter allele plays a role in driving the teratoma phenotype that appears after E15.5, it is likely acting in the regulation of germ cells prior to or just after their arrival in the gonad. Although we found that the protein was translated in HEK293 cells, it was present at much lower levels and bound RNAs much less efficiently than the wild-type allele. However, based on a single successful DO-RIP-seq assay, ~75% of the RNAs bound by DND1 also came down with DND1-Ter. Although this limited finding is in no way conclusive, it seems likely that DND1-Ter binds targets less efficiently, but could interfere with the ability of DND1 to regulate the stabilization or degradation of targets. Further experiments would be required to validate this finding.

**MATERIALS AND METHODS**

**Mice, timed matings and genotyping**  
*Dnd1Ter* mice were kindly provided by Dr Joseph Nadeau and maintained on a 129T2/SvEmsJ background. 129T2/SvEmsJ (hereafter referred to as ‘129T2’; www.jax.org/strain/002065) was predicted to be the closest living strain at The Jackson Laboratory (JAX) to the original 129/SvJ strain on which Ter arose. Because the formation of teratomas is a 129-strain-specific...
phenotype, to generate mutants in which germ cells were fluorescently tagged, Oct4-EGFP (originally on a mixed genetic background) (Yoshimizu et al., 1999) was back-crossed to 129T2-Dnd1Ter/− for nine generations, then crossed to 129T2-Dnd1Ter/− mutant females to generate 129T2-Dnd1Ter/−; Oct4-EGFP male and female heterozygotes. For timelapse matings, heterozygotes were intercrossed, females were checked for plugs and staged as day E0.5 if positive. For genotyping, tail DNA was extracted using standard methods and genotyped using the following primer sets: GFP-F, 5′-AAG TTC ATC TGC ACC G-3′; GFP-R, 5′-TCC TTG AAG ATG GTG CG-3′; Dnd1-F, 5′-GTA GTT CAG GAA CTC CAC TTG TG-3′; Dnd1-R, 5′-GCT CAA GTT CAG TAC GCA C-3′. Dnd1 Ter mice were genotyped by PCR using an annealing temperature of 62°C. The PCR product (145 bp) was digested overnight at 37°C with the restriction enzyme DdeI and run on a 4% agarose gel or 6% acrylamide gel. DdeI-mediated digestion of DNA was performed using Cufflinks pipeline (version 2.2.0) (Top-Hat, Cufflinks, Cuffmerge) was used to describe the magnitude of change and the significant enrichment/depletion of specific pathways using the Qiagen IPA database (Application build: 470319M; Content version: 43605602). DEGs included those that were differentially expressed: (1) between developmental stages (software.broadinstitute.org/gsea/) as described previously (Subramanian et al., 2005). Log2 ratio of classes was used to rank changes in gene expression between developmental stages as well as between wild-type and Dnd1 Ter/Ter samples. Normalized enrichment score (NES) and FDR values were used to describe the magnitude of change and the significant enrichment of the gene sets, respectively.

Ingenuity pathway analysis
Differentially expressed genes (DEGs) were assessed for statistical enrichment/depletion of specific pathways using the Qiagen IPA database (Application build: 470319M; Content version: 43605602). DEGs included those that were differentially expressed: (1) between developmental stages within a Ter genotype class; and (2) between Ter genotype classes at a specific stage. The cutoff for inclusion in the IPA analysis was set at a differential expression FDR<0.01.

Dnd1 DO-RIp-seq
DO-RIp-seq was performed as described previously (Nicholson et al., 2017). Briefly, 5-15 cm² plates of HEK293 cells were transfected with ∼35 µg/plate FLAG-DND1 or untagged DND1 (as a negative control), grown for 24 h, then harvested in PLB. Lysates were treated with 50 U/µg of Mnase for 5 min at 30°C and FLAG immunoprecipitation was performed. RNA from IPs was radiolabeled and run on a 10% TBE-Urea gel. RNA fragments from ∼25-75 nucleotides were excised and extracted from the gel. cDNA libraries were prepared using the NEBNext Multiplexing Small RNA Library Prep Set for Illumina with 14-17 rounds of amplification, following the manufacturer’s protocol with the exception that the 5′ RNA adapter was replaced with a custom 5′ adapter compatible with UMI (Unique Molecular Identifier) tagger (Kivioja et al., 2012) (RNA adapter sequence GUUCAGAGGUACAGGCCAGAUCNNNN). In addition to DND1, negative control IPs input samples were also prepared for
sequencing in a similar fashion using 17 rounds of amplification. An aliquot of the MNano-digested lysate prior to immunoprecipitation was set aside for generating input libraries. rRNA depletion was performed on input samples using the Epicenter Ribo-Zero Gold rRNA removal kit. Input RNA was size selected and libraries were prepared as described above for the immunoprecipitation samples. All experiments were performed as biological duplicates.

Libraries were sequenced using Illumina HiSeq 2500. Processing of data was performed as described by Nicholson et al. After removal of adapter and UMI sequences, reads were mapped to hg19 using TopHat2. Binding sites were identified using the binning and normalization procedure of Nicholson et al. (2017). IPVs were compared with input samples to identify sites enriched in DND1 immunoprecipitates. Reads with a low pass filter of >3 reads and >1.5-fold enrichment over input were considered DND1-binding sites.

Acknowledgements
We thank the teams in the FACS and Genomics Cores at Duke, especially Mike Cook, Nicolas Devos and Olivier Fedrigo, who helped with these experiments and their analysis. We are also grateful to Alex Bortvin, who provided information for the RepEnrich analysis; to Cindu Nicholson who provided technical advice for DO-RIP-seq experiments; to Joe Nadeau, who supplied the mice that found our Dnd1^{ter} colony; to past and present members of the Capel lab, especially Jordan Batchvarov, who contributed to the management of the strain over the last 10 years; and to Mike Czerwinski, who transferred bioinformatics expertise to J.A.G.

Competing interests
The authors declare no competing or financial interests.

Author contributions

Funding
This work was supported by the National Institutes of Health (GM087500 to B.C.); by Bridge Funding from Duke University Medical Center (to B.C.); and by the National Cancer Institute (CA157268 to J.D.K.). Deposited in PMC for release after 12 months.

Data availability
The raw RNA-Seq and DO-RIP-seq datasets have been deposited in GEO as a single unified SuperSeries with accession number GSE132719.

Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.175950.supplemental

References
Agueiro, T., Jin, Z., Chorghade, S., Kalsotra, A., King, M. L. and Yang, J. (2017). Maternal Dead-end 1 promotes translation of nanos1 by binding the eIF3 colony; to past and present members of the Capel lab, especially Jordan Batchvarov, who contributed to the management of the strain over the last 10 years; and to Mike Czerwinski, who transferred bioinformatics expertise to J.A.G.

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Fig. S1. Examples of genes detected in the Dnd1<sup>Ter/Ter</sup> transcriptome data that are specific to Sertoli or Leydig cell lineages in the gonad based on the Jameson data (2). Typically, expression of these genes spikes in the E14.5 Dnd1<sup>Ter/Ter</sup> data (orange), a stage when Dnd1<sup>Ter/Ter</sup> germ cell numbers are reduced and expression of Oct4-GFP declines, which may have led to errors in threshold settings during FACS. Graphs of transcriptome data from each cell type in the gonad performed at E11.5, E12.5 and E13.5 are shown for comparison. The germ cell lineage is green; supporting cells are blue; interstitial/stromal cells are purple; endothelial cells are red.
Fig. S2. Male germ cells do not acquire a female (or meiotic) identity, but retain expression of pluripotency genes shared with female germ cells. (A) Heat map analysis showing that Dnd1Ter/Ter male germ cells retain a profile very similar to wild type at E12.5 and E13.5 with respect to genes that are specifically depleted in male germ cells, and genes that are specifically enriched. (B) Similarly, heat maps comparing expression of female-specific genes in XY Dnd1+/+ to XY Dnd1Ter/Ter samples show few changes for depleted or enriched genes at E12.5 or E13.5. (C) A cumulative distribution plot of the log2 fold change between mutant male germ cells and wild type male germ cells at E13.5 for female germ cell enriched (green) or depleted (red) genes. Genes typically enriched (green) or depleted (red) in female germ cells show no clear bias for the direction of change in mutant germ cells. (D) Genes specifically upregulated as female germ cells enter meiosis (Stra8 and Sycp3) were not activated by E14.5 in Dnd1Ter/Ter mutants. Graphs to the right show expression of the gene in all gonadal lineages (green broken line = XX germ cells; green solid line = XY germ cells; data from (2)). (E) Rhox9 and Rhox6, which normally become specific to XX germ cells by abrupt down-regulation in male germ cells, show a delay in down-regulation similar to other pluripotency genes.
Fig. S3. Dnd1^{Ter/Ter} germ cells show no evidence of lineage infidelity between E12.5-E14.5.

Despite the failure to activate many chromatin regulators in Dnd1^{Ter/Ter} mutants, (A) expression of bivalent genes grouped by category (cytoplasmic signaling proteins, enzymes, transcription factors, secreted proteins, transmembrane proteins, and other) was not elevated, and (B) with the exception of Hpcal1, neither neuronal genes, nor other transcription factors associated with somatic cell differentiation (Pax6, Eomes, Olig3, Otx2, and Lhx1) were significantly elevated in mutants.
Fig. S4. Repetitive elements in general do not exhibit aberrant activity. (A) A survey of repetitive element distribution organized by class. Outer ring: class distribution of elements represented in reads at E14.5 (Red=DNA transposons; Blue=LINEs; gold=LTRs; green=SINEs; Magenta=satellite; Orange=simple repeats; Dark Green=snRNAs; Purple=other). Inner circle divides specific classes into reads with significant differences between mutant and wildtype and reads without significant differences between mutant and wildtype (bold colors: FDR<0.05, significant; pale colors: FDR>0.05). (B) Waterfall plots of Log Fold Change (LogFC) between mutant and wildtype in each transposable element class (LINEs, LTRs, and DNA transposons) with FDR<0.05 (significant). LogFC shown to left of plots; FDR shown by orange triangle placement relative to right scale on each graph.
Fig. S5. In general, miRNAs did not show activation based on downstream target analysis. (A) Although DND1 is believed to block degradation of zebrafish miR430 targets, with the exception of Lats2, targets of the orthologous miRNA in mice (miR320) are not down-regulated in mutants as predicted. (B) Waterfall graphs of Ingenuity Pathway Analysis (Qiagen) of miRNA activation based on expression changes in predicted targets at E12.5-E13.5 (top) and E13.5-E14.5 (bottom) for wild type (blue) and mutant (orange) samples. Only miRNA pathways with an activation Z-score of >2 or <-2 (left y-axis) in at least one transition period for mutant or wild type data are shown. P-values are plotted on the right Y-axis and represented as diamonds.
Fig. S6. Four RBPS closely related to DND1 are expressed in germ cells. Four of the five RBPs (*Rbm47, Rbm46, Syncrip, Hnrnpr, but not A1cf*) related to DND1 (Fig. 3) are expressed in male germ cells during the transcriptome timecourse.
Figure S7. Levels of the Dnd1-Ter transcript decline relative to wild type in mutants, and the flag-tagged allele is poorly represented relative to the wild type allele when over-expressed in HEK293 cells. (A) Dnd1-Ter transcript level in mutants is similar at E12.5, but >4-fold lower than wild type by E14.5. (B) While the flag-tagged mutant protein is expressed in HEK293 cells based on Western analysis, it is detected at much lower levels. (C) Immunoprecipitation of the tagged mutant protein relative to input yields much lower levels of DND1-Ter than wild type DND1. (D) Autoradiography of the RNA associated with DND1-Ter vs the wild type allele shows no difference above background.
Fig. S8. Other chromatin regulators that are not expressed in HEK293 cells, thus did not map as targets of DND1 in RIP assays, fail to be activated in mutants. Nine additional chromatin regulators (Brdt, Six1, Ezh1, Zmym3, Kdm5b, Kat2a, Smarcb1, and Prmt5) are all expressed at lower levels in Dnd1^Ter/Ter mutants likely reflecting a failure to activate the male pathway.
### Table of Pathways and P-values

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<thead>
<tr>
<th>Pathway</th>
<th>P-value</th>
<th># Affected Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Death and Survival</td>
<td>$1.05 \times 10^{-4}$ – $3.21 \times 10^{-22}$</td>
<td>433</td>
</tr>
<tr>
<td>Cellular Growth and Proliferation</td>
<td>$1.06 \times 10^{-4}$ – $5.18 \times 10^{-20}$</td>
<td>482</td>
</tr>
</tbody>
</table>
Fig. S9. Cell death and cell cycle pathways are strongly affected in $Dnd1^{Ter/Ter}$ mutants. (A) Ingenuity pathway analysis identified cell death (433 genes) and cell cycle (482 genes) pathways as the most strongly affected in $Dnd1^{Ter/Ter}$ mutants. (B) Network diagram centered on $Tp53$, depicting the relationship of many cell death and survival genes. (C) Network diagram centered on $Ccne1$, depicting the relationship of many cell cycle genes, most of which are up-regulated.
Fig. S10. Expression of genes in the mTor, Hippo and Bmp/Nodal pathways are altered in Dnd1Ter/Ter mutants. Expression of other members of the (A) mTor, (B) Hippo and (C) Bmp/Nodal pathways from Fig. 5 are misregulated in mutants with some mapping as transcript targets of DND1 (Fig. 5A).
Table S1. RNAseq gene expression graphing. We provide an excel file with (A) the RPKM values used in our analysis and (B) the graphic format used to display gene expression in wild type and $Dnd1^{Ter/Ter}$ mutants over time. The user can copy any row from (A) with all values into row 2 of (B) to generate the graph for that gene.

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Table S2. Lists of genes significantly up-regulated or down-regulated in $Dnd1^{Ter/Ter}$ mutants at E12.5, E13.5 and E14.5, ordered from the highest to the lowest log2-fold change.

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Table S3. The list of changed genes defined by GSEA as part of the “DPPA4 Targets”.

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Table S4. List of genes that are (A) up-regulated or (B) down-regulated in Nanos2 mutants, annotated for activity in Dnd1Ter/Ter mutants.

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Table S5. (A) List of all genes (expressed or not expressed in HEK293 cells), indicating genes identified in DO-RIP and/or PAR-CLIP (1). (B) List of all binding sites enriched in DO-RIP-seq. (C) List of genes with reproducible 3'UTR binding sites enriched in both DO-RIP-seq experiments. This table was used to label targets in Figure 5A. (D) List of genes unique to Ruthig study, unique to Yamaji study, or identified in both studies.

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Supporting Materials - References
