DAZL Limits Pluripotency, Differentiation, and Apoptosis in Developing Primordial Germ Cells

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SUMMARY

The scarcity of primordial germ cells (PGCs) in the developing mammalian embryo hampers robust biochemical analysis of the processes that underlie early germ cell formation. Here, we demonstrate that DAZL, a germ cell-specific RNA binding protein, is a robust PGC marker during in vitro germ cell development. Using *Dazl*-GFP reporter ESCs, we demonstrate that DAZL plays a central role in a large mRNA/protein interactive network that blocks the translation of core pluripotency factors, including *Sox2* and *Sall4*, as well as of *Suz12*, a polycomb family member required for differentiation of pluripotent cells. Thus, DAZL limits both pluripotency and somatic differentiation in nascent PGCs. In addition, we observed that DAZL associates with mRNAs of key Caspases and similarly inhibits their translation. This elegant fail-safe mechanism ensures that, whereas loss of DAZL results in prolonged expression of pluripotency factors, teratoma formation is avoided due to the concomitant activation of the apoptotic cascade.

INTRODUCTION

In the mouse, germline specification begins around E6.25, when the expression of PR domain proteins (PRDM1 and PRDM14) mediates the transcriptional suppression of the somatic differentiation program in the nascent primordial germ cells (PGCs) (Kurimoto et al., 2008; Yamaji et al., 2008). Shortly after the specification period, the importance of posttranscriptional regulation in germ cell maintenance becomes evident. For example, in mice lacking DND1 or NANOS3, both of which are germ cell-specific RNA binding proteins, PGCs are gradually lost by apoptosis starting at E8.5, when PGCs start to migrate toward the future gonads (Youngren et al., 2005; Suzuki et al., 2008). Upon arrival in the gonad, DAZL (*D*eleted in *a*zoospermia-*l*ike), a germ cell-specific RNA-binding protein, is essential for developing PGCs (Lin and Page 2005), and loss of Dazl expression again results in apoptosis of the postmigratory germ cells. The DAZ (*D*eleted in *A*zoospermia) gene family is composed of the germ cell-specific genes *Boule*, *Dazl*, and *DAZ*. The encoded proteins all contain RNA recognition motifs (RRM) in the N-termi-

nal domains, which presumably interact with target RNAs, and one or multiple DAZ repeats in the C-terminal domain, of which the function is still unknown. *Dazl* is conserved in all vertebrates, and the transcripts of *Dazl* are germ plasm components in both *Xenopus* (Houston et al., 1998) and zebrafish (Hashimoto et al., 2004). Although a germ plasm analog was never identified in mammalian PGCs, loss of *Dazl* expression results in infertility in both sexes in mice, with germ cell loss during development and a final block at meiosis (Ruggiu et al., 1997; Schrans-Stassen et al., 2001). In the C57Bl/6 background, apoptosis of *Dazl* knockout germ cells is observed at the early gonocyte stage (Lin and Page 2005).

Given the importance of DAZ family proteins in germ cells, much effort has been invested in elucidating the molecular mechanisms of *Dazl* function. Lin and colleagues demonstrated that DAZL is a meiosis-promoting factor in developing germ cells (Lin et al., 2008). Indeed, in the absence of DAZL, the germ cells fail to develop beyond the PGC stage as shown by continued expression of pluripotency markers. These findings gave rise to the idea that DAZL is a "licensing factor" that is required for

PGC sexual differentiation (Gill et al., 2011). However, the mechanism by which DAZL promotes meiotic entry remains unclear. To elucidate the function of DAZL in germ cell development, several groups have identified mRNA binding partners in coimmunoprecipitation experiments (Fox et al., 2005; Reynolds et al., 2005; Zeng et al., 2008) and yeast three-hybrid assays (Venables et al., 2001). Potential mRNA targets include *Mvh* (Reynolds et al., 2005), *Scp3* (Reynolds et al., 2007), and *Tex19.1* (Zeng et al., 2009). In most of these studies, DAZL was shown to function as a translational enhancer. Yet, the ablation of *Tex19*, *Mvh*, or *Scp3* in mice results in fertility phenotypes that are patently less severe and arise much later in development than the *Dazl* knockout phenotypes, suggesting that DAZL may have additional roles during the PGC stage of mammalian gametogenesis.

Unfortunately, exploration of the biochemical mechanisms that underlie germ cell specification and early PGC formation in the mammalian embryo is hampered by the scarcity of cells at these early embryonic time points. In vitro derivation of PGCs from embryonic stem cells (ESCs) allows the generation of sufficient cell numbers to perform robust biochemical analysis of protein-protein and protein-mRNA interactions (Hübner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Hayashi et al., 2011). To explore the role of DAZL during PGC development, we have generated a *Dazl*-GFP reporter ESC line that allows the prospective isolation and biochemical analysis of in vitro developing PGCs. Using this in vitro assay, as well as conformational analysis in vivo, we demonstrate that DAZL acts as a translational repressor to simultaneously suppress pluripotency, somatic differentiation, and apoptosis in nascent PGCs.

RESULTS

A *Dazl*-GFP Reporter Marks Developing PGC-like Cells In Vitro and PGCs In Vivo

In order to study the function of DAZL in the germ cell context, we generated a recombinant BAC clone in which GFP-V5 was placed immediately in front of the stop codon, thus creating a *Dazl*-GFP-V5 fusion that is controlled by Dazl promoter and enhancer sequences as well as posttranscriptional regulatory sequences present in the untranslated regions (Figures 1A and 1B). Transgenic mice were generated by blastocyst transfer of ESCs and subsequent crossbreeding of chimeras. *Dazl*-GFP transgenic mice are normal in gross appearance and are fertile, and the expression of *Dazl*-GFP is confined to the germline in all developmental stages examined. During embryonic development, GFP+ cells are found in hallmark patterns of male and female germ cells in the gonad (Figure 1C), whereas GFP

expression is absent in the rest of the embryo at this stage. In adult mice, *Dazl*-GFP is detected only in the testicular germ cells and in oocytes (data not shown). A previous study by Nicholas et al. describes in which GFP is expressed under control of a 1.7 Kb *Dazl* promoter region (Nicholas et al., 2009). Unfortunately, this reporter did not recapitulate early *Dazl* expression, as *Dazl*-GFP expression could only be observed after E17.5. Possibly, this promoter-based reporter lacks essential regulatory elements for the early expression of *Dazl* in developing PGCs. Our *Dazl*-GFP reporter includes all 5′ and 3′ regulatory elements, reliably reports endogenous *Dazl* expression, even during early PGC development.

The derivation of PGC-like cells in vitro, through differentiation of ESCs, allows biochemical analyses of the molecular pathways during mammalian embryonic germ cell development (reviewed in Ko et al., 2010). To test if the *Dazl*-GFP reporter is suitable for this purpose, we characterized the *Dazl*-GFP+ cells during germ cell differentiation of ESCs. In vitro, the *Dazl*-GFP reporter gene is expressed at low levels in 5%–10% of embryonic stem cells under standard LIF/MEF conditions. Similar heterogeneous expression of germ cell and pluripotency markers in ESCs was previously reported for *Stella* (Payer et al., 2006), *Nanog* (Singh et al., 2007), *Zfp42* (Carter et al., 2008), and *Zscan4c* (Zalzman et al., 2010). Upon ESC differentiation toward PGC-like cells, *Dazl*-GFP expression rapidly wanes and at day 4 of differentiation there are almost no GFP-expressing cells present in the culture (Figure 1D). Starting from day 4 a Dazl-GFP-positive cell population re-emerges and at day 12 of differentiation, typically 50% of the cells are *Dazl*-GFP positive and express the reporter at high levels (Figure 1D). When *Stella*-GFP reporter ESCs (Payer et al., 2006) are differentiated under the same conditions, we observed a similar decline of *Stella*-GFP-positive cells during the first 2 days of ESC differentiation as seen with the Dazl-reporter, followed by transient emergence *Stella*-GFP-positive cells (Figure 1D). These results are in line with the expression of *Stella* and *Dazl* in the early embryo, where *Stella* transiently marks early PGCs in the proximal epiblast at E7.5–E8.5 and is downregulated upon arrival at the gonads by E11.5 (Payer et al., 2006). In contrast, *Dazl* expression is initiated during PGC migration and continues to be expressed in developing germ cells up to the initiation of meiosis. Thus, the expression of *Stella*- and *Dazl*-reporter genes during in vitro differentiation recapitulates the gene expression changes observed during in vivo PGC development.

Gene expression microarray analysis showed upregulation of germ cell-specific transcripts in the in vitro-derived PGCs from both *Stella*-GFP and *Dazl*-GFP ESCs (Figure 1E and Figure S1 available online). In line with these findings, *Stella*+ in vitro PGCs express markers of early PGC specification, including *Dnd1*, *Prdm1*, and *Nanos3*. In contrast, the
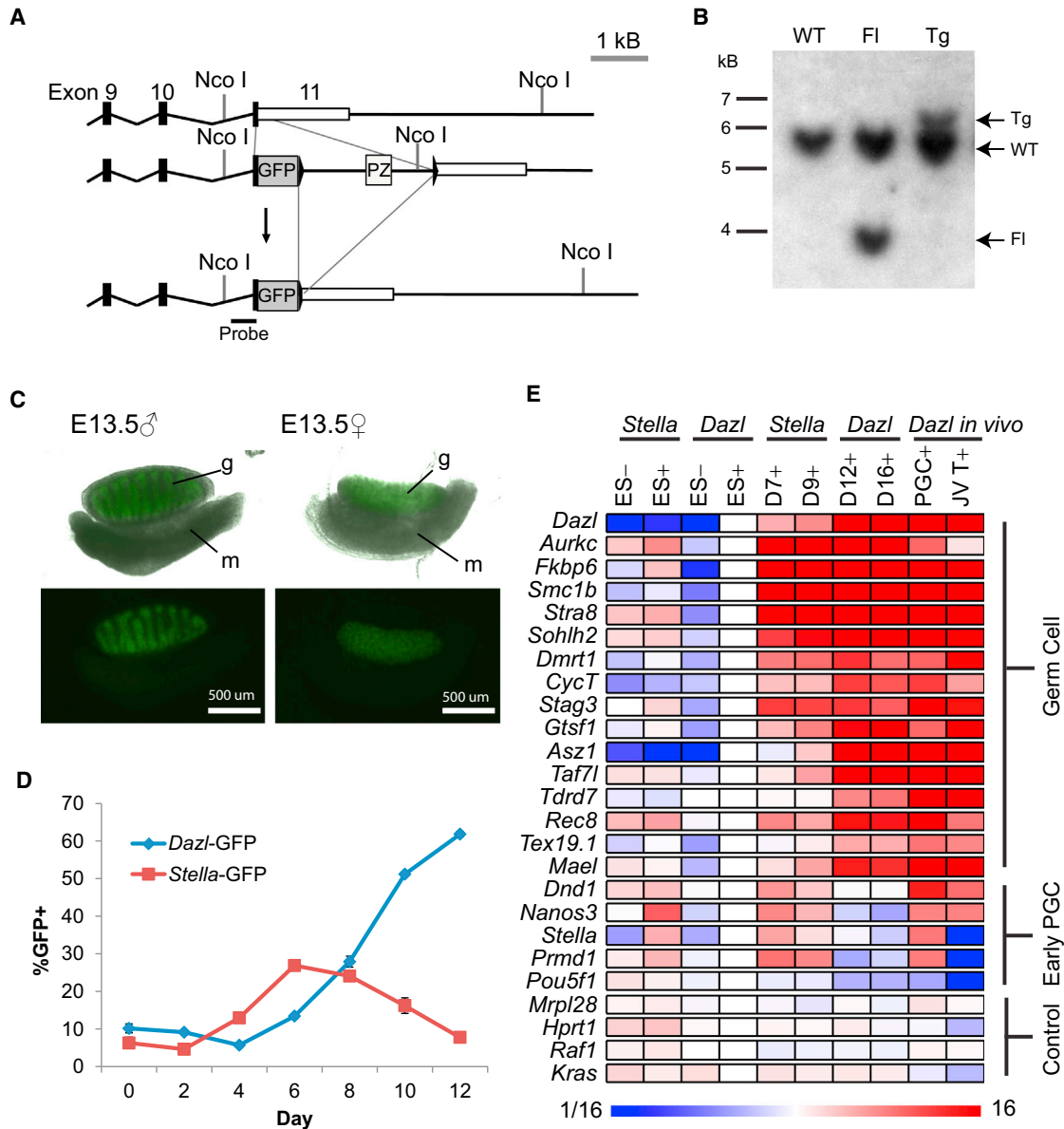
Figure 1. Generation of the *Dazl*-GFP-V5 Fusion Reporter Mouse

(A) Schematic view of *Dazl* targeting strategy. The *Dazl* stop codon was replaced with GFP-V5 coding sequence and a floxed puromycin resistance cassette by BAC recombineering in *E. coli*. The resulting floxed transgenic BAC was used to generate transgenic ES lines and chimeric mice. The floxed cassette was excised in vitro by transient transfection of an expression construct containing CRE-IRES-*tdtomato* and in vivo by mating with CMV-CRE mice (Jackson Laboratory, B6.C-Tg[CMV-cre]1Cgn/J).

(B) Southern blot analysis of mouse genomic DNA. A probe against a 500 bp region in the 3′ end of intron 10 was used to detect fragments flanked by NcoI sites in each genotype. WT, wild-type; Floxed, transgenic floxed; Tg, transgenic *Dazl*-GFP (CRE excised). Fluorescent microscopy of embryonic gonads (g) with mesonephros (m) attached from *Dazl*-GFP-V5 reporter mice.

(C) Fluorescence image of E13.5 gonads of male and female Dazl-GFP embryos (bottom panels) and the GFP signal superimposed onto bright-field image (top panels). Note strong GFP fluorescence in the germ cells (g), whereas GFP is absent from interstitial cells in the gonads and the attached mesonephros (m).

(D) Comparison of *Stella*-GFP and *Dazl*-GFP expression during in vitro differentiation. The reporter cell lines were plated in differentiation condition at day 0, and the percentages of GFP⁺ cells in culture were monitored by FACS analysis over 12 days. Data are represented as mean ± SEM. n = 2 biological replicates for each cell line.

(legend continued on next page)

transcriptome of *Dazl*⁺ PGCs is reflective of late-stage germ cell development, including the expression of *Gtsf1*, *Maelstrom*, *Tdrd7*, *Tex19*, *Taf7l*, and *Rec8*, all of which are absent in ESCs (Figures 1E and S1). Thus, at the transcriptional level, the emerging *Dazl*⁺ cells are PGC-like cells. Juvenile testis was included as control for the expression levels of germ cell-specific genes in diploid germ cells, because at this postnatal stage meiosis has not been initiated yet. As shown, germ cell-specific genes are expressed at comparable levels in in vitro PGC-like cells and juvenile germ cells (≤2 weeks old) (Figure 1E).

### DAZL Localizes in P-Bodies in Developing Germ Cells but Not in ESCs

The ability to generate *Dazl*⁺ PGC-like cells in vitro provided us with a unique opportunity to generate sufficient material for biochemical analysis of this otherwise rare cell population. We focused in these assays on day 11–13 of in vitro differentiation because at these time points *Dazl* was consistently expressed at high level in the in vitro PGC-like cells. Aside from reporting the expression of *Dazl*, the GFP-*Dazl* fusion gene allowed us to analyze the subcellular localization of DAZL during germ cell differentiation in vivo and in vitro. Previous studies showed that DAZL is localized to the cytoplasm in porcine oocytes and in murine testis (Ruggiu et al., 1997; Liu et al., 2009). In our transgenic system, the *Dazl*-GFP fusion protein was also cytoplasmic in adult spermatogonia, PGCs and in vitro PGCs, distributed both diffusely as well as in granules (Figures 2 and S2A). The *Dazl*-GFP granules appear to be heterogeneous, because they partially colocalizes with RNA processing body (P-body) markers EDC4 (GE-1) and DCP1a (Figures 2 and S2A). We also monitored DAZL localization in live PGC-like cells generated in vitro. As shown in Figure S2B, DAZL reveals a punctate staining in live cells as well, demonstrating that the granular localization of DAZL is not the result of the fixation and/or immunofluorescent staining procedures. Although P-bodies are also present in ESCs, *Dazl*-GFP does not localize in granules in undifferentiated ESCs but instead is homogeneously distributed in the cytoplasm (Figure S2C). DAZL granular localization is not the result of the fusion with GFP but, rather, depends on the presence of the C-terminal DAZ-domain of the protein (Figures 2E and S2D). Together, our results demonstrate that DAZL dynamically colocalizes with P-body markers in cytoplasmic granules, specifically in PGC-like cells in vitro and PGCs in vivo.

Protein Binding Partners of DAZL Granules

To further interrogate the role of DAZL during germ cell differentiation, we identified DAZL-interacting proteins by affinity capture-mass spectrometry from in vitro PGCs (Figures 3A and S3). *Dazl*-GFP-interacting proteins were coimmunoprecipitated using anti-V5 antibody and resolved by 2D gel electrophoresis. Immunoprecipitations using the same antibody from wild-type ESCs not expressing the *Dazl*-GFP fusion protein served as negative control. Specific DAZL-interacting proteins were excised from the gel and identified by mass spectrometry analysis. A list of proteins specifically associating with *Dazl*-GFP is provided in Table S1. Figure 3A shows the functional analysis of the DAZL protein complex using STRING 9.0 (http://string-db.org/), a database of known and predicted protein interactions that include direct (physical) and indirect (functional) associations (Snel et al., 2000; Smoot et al., 2011; Baltz et al., 2012; Castello et al., 2012). As shown, the identified proteins are predominantly RNA-binding proteins. In fact, 72% of the DAZL-interacting proteins were recently identified as RNA binding proteins in poly(A) pull-down experiments from human somatic cell lines (Baltz et al., 2012; Castello et al., 2012), suggesting the highly germ cell-specific DAZL associates may act to add a germ cell-specific function to a more broadly expressed RNA regulatory network. Indeed, many of the DAZL-interacting proteins have been reported as components of the IGF2BP1 RNP complex (Jønson et al., 2007; Weidensdorfer et al., 2009). The IGF2BPs (Zipcode binding proteins, ZBPs) are RNA binding proteins known to form RNP structures that function in translational inhibition or RNA stabilization in neurons, fibroblasts, and other cell lines (Hüttelmaier et al., 2005; Weidensdorfer et al., 2009). Because IGF2BPs have been shown to interact with other proteins in an RNA-dependent fashion (Weidensdorfer et al., 2009), we next analyzed whether the DAZL protein complexes are also RNA dependent. As demonstrated in Figure 3B, DAZL interactions with IGF2BP1 and other known components of the IGF2BP1 RNP are abolished by RNase treatment of the sample prior to coimmunoprecipitation, suggesting that the proteins either complex through common RNA targets or interaction with RNA targets is required for the proteins to adopt a specific conformation required for protein-protein interaction.

In addition to components of the IGF2BP1 RNPs, we also identified Fragile-X Mental Retardation Protein (FMRP) and two of its related proteins, Fragile X Related 1 and 2

(E) Gene expression microarray analysis of in vitro PGCs, in vivo PGCs, and germ cells from juvenile testis. The expression profiles of germ cell signature genes in in vitro germ cells from two different reporter cell lines. *Stella*, *Stella*-GFP BAC reporter cell line; *Dazl*, *Dazl*-GFP reporter cell line; +/−, FACS-sorted GFP⁺/GFP⁻ cell populations. PGCs were *Dazl*-GFP⁺ cells sorted from E13.5 gonads, and testicular germ cells were *Dazl*-GFP⁺ cells sorted from juvenile male mice (<2 weeks old).
See also Figure S1.

Testis   E12.5♂   *in vitro* PGC

GE-1
Dazl-GFP
DAPI

10 µm
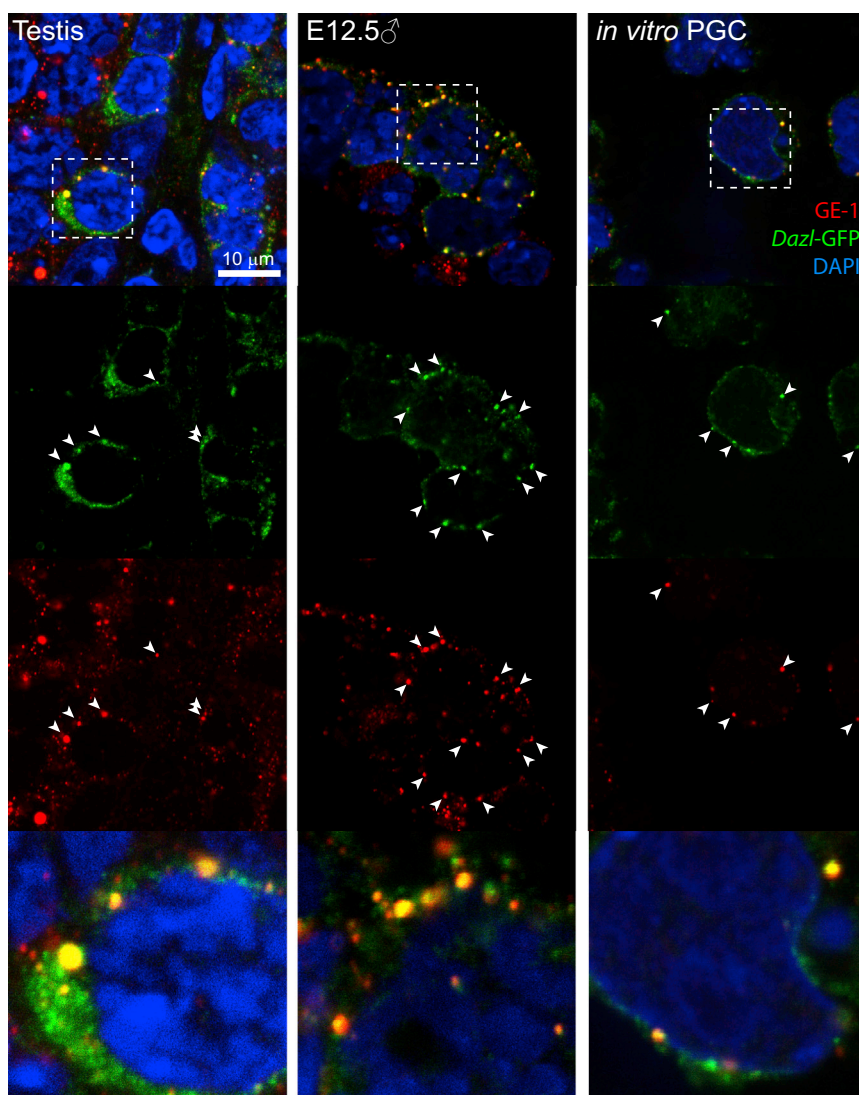
**Figure 2. Subcellular Localization of *Dazl*-GFP in Germ Cells**
Granular localization of *Dazl*-GFP. Left panel, adult *Dazl*-GFP testis cryosection; middle panel, PGCs from male E12.5 embryos, dissociated and cytospun; right panel, day 13 in vitro *Dazl*-GFP⁺ PGCs, FACS sorted, and cytospun.

(FXR1 and FXR2), in the co-IP complex. Interestingly, FMRP has been shown to interact directly with IGF2BP1 (Rackham and Brown 2004) and has an established role in RNA transport and translational inhibition in neuronal dendrites. Using immunofluorescence microscopy, we further analyzed the subcellular localization of these proteins in vivo and as shown in Figure 3C, *Dazl*-GFP colocalizes with IGF2BP1, FXR1, and FMRP in cytoplasmic granules in adult testicular germ cells. The co-IP of in vitro PGC-like cells and cell-imaging results of germ cells in vivo together indicate that DAZL locates to RNA-protein granules together with known inhibitors of mRNA translation.

Identification of DAZL-Interacting mRNAs in PGCs
Thus far, several labs have suggested DAZL functions as an enhancer of mRNA translation in the germline (Collier et al., 2005; Reynolds et al., 2005, 2007; Chen et al., 2011). We were therefore surprised to find DAZL interaction with RNP structures that are known to regulate translational inhibition. To uncover the molecular pathways downstream of DAZL in the PGCs and a possible role for DAZL in stabilizing specific mRNAs in the germline, we first analyzed changes in global transcription upon *Dazl* knockdown. For this, we utilized *Oct4*-GFP reporter ESC lines, because the *Dazl*-small hairpin RNAs (shRNAs) would also affect the expression of our *Dazl*-GFP reporter. *Oct4*-GFP ESCs were infected with shRNA lentiviruses targeting *Dazl*. In the in vitro PGCs, we observed >95% knockdown of *Dazl* mRNA with two different short hairpin RNAs. Global gene expression analysis of the *Dazl* knockdown revealed very limited changes in the transcriptome in in vitro PGC-like cells (Figure S4). Only one mRNA, *Asb9*, was found to be significantly affected by *Dazl* knockdown
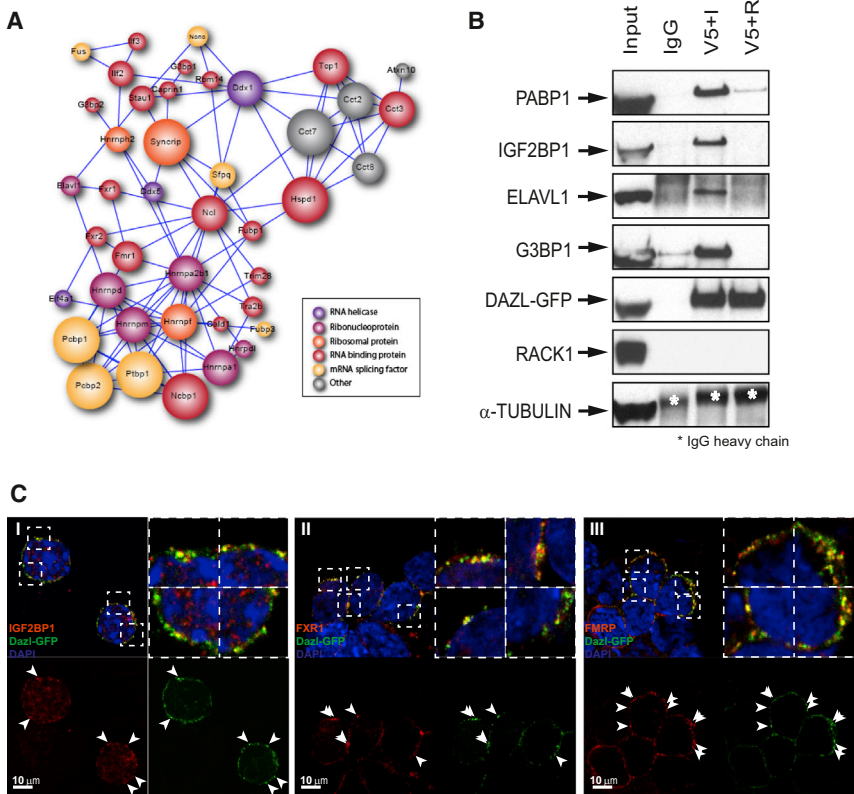
Figure 3. A Network of DAZL-Interacting Protein Partners

(A) DAZL-interacting proteins identified by MS were subjected for functional analysis using STRING 9.0 (http://string-db.org/). STRING is a database of known and predicted protein interactions, which include direct (physical) and indirect (functional) associations; they are derived from four main sources: genomic context, high-throughput experiments, coexpression, and previous knowledge (Snel et al., 2000). Protein links found by STRING were loaded in Cytoscape 2.8 (Smoot et al., 2011) as binary interactions. An organic layout was then applied to the resulting network. The size of the nodes is displayed as a function of the number of interaction present in each node. Protein nodes were further functionally categorized using Panther Classification System (http://www.pantherdb.org).

(B) Immunoblot analysis of DAZL-interacting proteins from day 11 differentiated culture. IgG, normal mouse IgG control; V5+I, anti-V5 IP with RNase inhibitor; V5+R, anti-V5 IP with RNase A treatment.

(C) The *Dazl*-GFP cells were sorted from adult testes, cytospun, and stained with anti-IGF2BP1 (panel I), FXR1 antibodies (panel II), or FMRP antibodies (panel III) for confocal imaging. White arrows denote foci of colocalization.

(Figure S4), yet further analysis described below revealed that this gene is not directly downstream of DAZL and therefore likely a secondary consequence of the loss of *Dazl* expression. These results demonstrate that loss of DAZL does not profoundly affect the stability of specific RNAs in in vitro developing PGC-like cells.

Next, we identified the specific mRNA targets of DAZL in the developing PGC-like cells. Using standard RNA-IP protocols as described in Experimental Procedures, we isolated RNAs associated with *Dazl*-GFP-V5 from in vitro PGCs and identified them using gene expression microarrays. For comparison, we also analyzed the RNA transcripts coimmunoprecipitated with *Dazl*-GFP-V5 from juvenile testis (postpartum day 14), which contain mostly diploid germ cells at this stage. The DAZL-interacting mRNA candidates were filtered based on folds of enrichment in IP compared to input and mock IP (immunoglobulin [Ig] G or anti-PABP1) and on the basal expression levels in the input cell lysates, using cutoffs at 3-fold in native condition IPs and 4-fold in UV-crosslinked IPs and signal/noise >10. Importantly, *Asb9* transcript, the sole gene of which the expression was altered upon loss of *Dazl* expression, was

not enriched in any of the DAZL coprecipitants, indicating that *Asb9* is not a direct mRNA target of DAZL and that the effect of DAZL on *Asb9* expression is therefore indirect. We used both native IP conditions and UV crosslinking to exclude the possibility of experimental artifact that can occur with either method alone. Under native conditions the wash conditions are less stringent, which can result in false-positives. UV crosslinking prior to the immunoprecipitation allows stringent wash conditions with strong detergents but can yield false-positives due to the UV-crosslinking process itself. Juvenile testes were included as a control for DAZL-mRNA targets in vivo. We therefore identified mRNA targets that were specifically enriched in both the UV-crosslinking experiments as well as native IP conditions in two independent experiments in vitro PGC-like cells and narrowed down potential DAZL target RNA transcripts to 453 genes. Gene Ontology analysis of the targets revealed overrepresentation of genes involved in RNA processing, RNA splicing, protein degradation, and transcriptional regulation (Table S2). One hundred twenty of these genes were also identified in RNA-IPs from *Dazl*-GFP juvenile testes. We conducted in silico
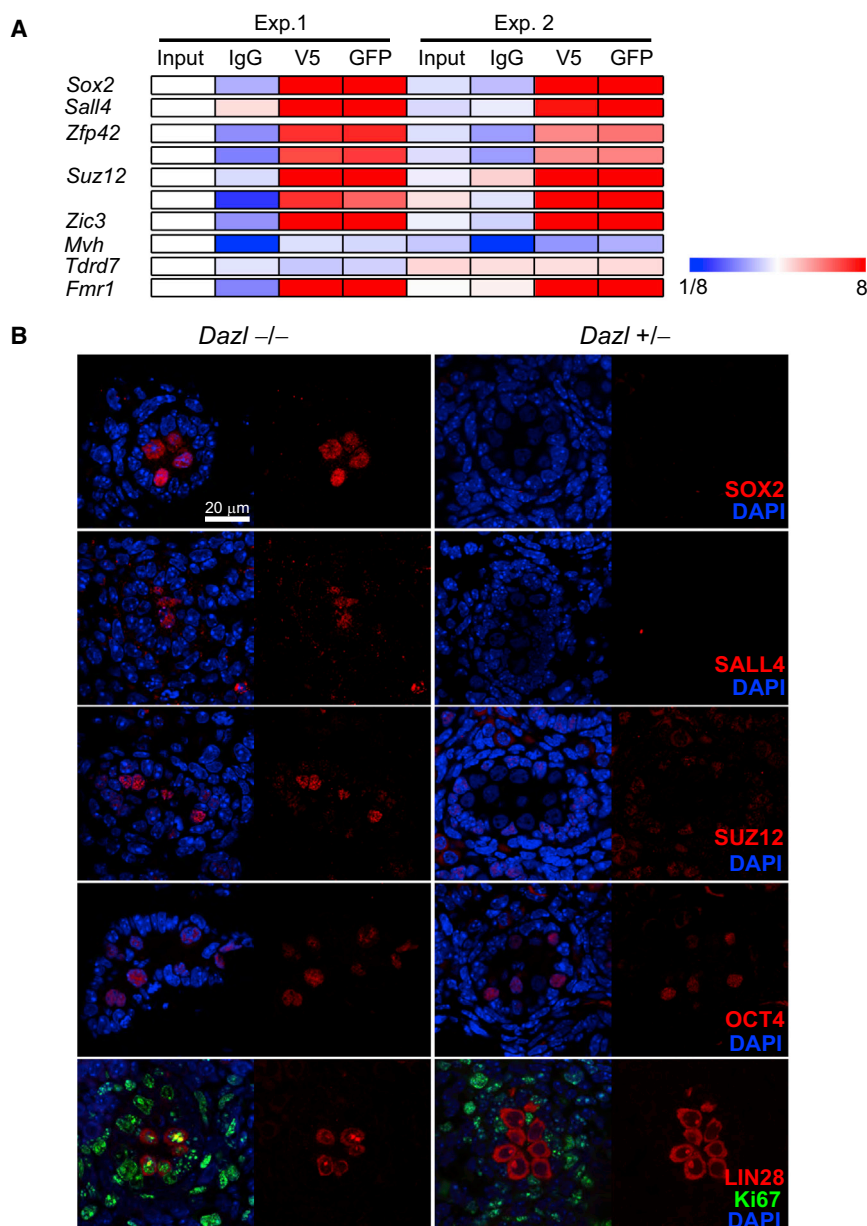
Figure 4. DAZL Regulates Pluripotency Genes by Translational Inhibition

(A) Identification of DAZL-interacting mRNAs. Microarray analysis of two independent native RNA-IP experiments from in vitro PGCs are shown. Input: total RNA in lysate; IgG, mock IP with normal mouse IgG; α-GFP, RNA-IP with rabbit-anti-GFP; α-V5, RNA-IP with mouse-anti-V5. All genes shown are expressed at signal/noise >20 in input RNA control.

(B) Expression of pluripotency genes in the absence of DAZL. Immunofluorescence staining of SOX2, SUZ12, SALL4, and Ki67 on cryosections of E16.5 embryonic testes from $Dazl^{-/-}$ and $Dazl^{+/-}$ littermates are shown.

searches for sequence motifs in the RNAs coprecipitated with *Dazl*-GFP. The only motif identified with significant enrichment is the 8-mer UUUGUUUU located in 3′ UTRs of the Dazl-associated transcripts (p = 3.55 × 10⁻¹⁵). Because this sequence motif is consistent with those identified in previous reports, we considered target genes containing this motif with higher priority.

DAZL Directly Suppresses Specific Pluripotency Gene Expression

Among the mRNA targets of DAZL identified by RNA-IP, we noticed a panel of genes known to be important for the maintenance of pluripotency in embryonic stem cells.

These include *Sox2*, *Sall4*, *Suz12*, *Zfp42*, and *Zic3* (Figure 4A), all of which contain the archetype *Dazl*-binding motif in their 3′ UTRs of both mouse and human transcripts (Table S3). To further explore the functional role of the DAZL interaction with these mRNA targets, we analyzed the expression of these genes by immunofluorescence in E16.5 embryonic testes of $Dazl^{-/-}$ mice (Ruggiu et al., 1997; Lin and Page 2005) and heterozygous littermates. In the *Dazl* knockout gonads, SOX2, SALL4, and SUZ12 proteins are aberrantly expressed in the residual germ cells, whereas these proteins are undetectable in germ cells from $Dazl^{+/-}$ littermates (Figure 4B). In contrast, OCT4, another key pluripotency gene, but not a DAZL
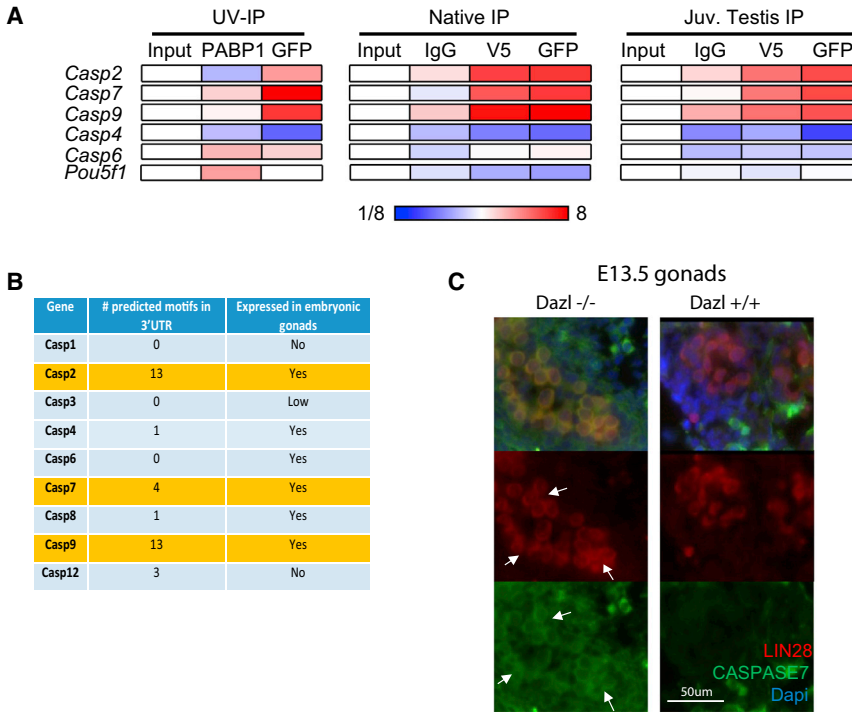
Figure 5. DAZL Associates with Transcripts of Proapoptotic Genes

(A) Left: RIP-microarray experiment from UV-crosslinked in vitro PGC culture. Input RNA and PABP RNA-IP were used as negative controls. Middle: RIP-microarray experiment from in vitro PGC culture without crosslinking. Antibodies against GFP and V5 were used to immuniprecipitate DAZL, and input RNA and normal mouse IgG were used as negative controls. Right: RIP-microarray experiment from juvenile (P14) *Dazl*-GFP testis lysate without crosslinking. Results for *Pou5f1* are also shown for comparison. n = 2 for each IP.

(B) Schematic analysis of putative DAZL-binding sites in transcripts of Caspase family members.

(C) Immunohistochemistry analysis of CASPASE7 expression (green) in embryonic gonads of E13.5 mice. Left panel, *Dazl*$^{-/-}$ mice; right panel, heterozygous littermates. LIN28 staining (red) was used to identify developing PGCs. Arrows show areas with both LIN28 and CASPASE7 staining.

mRNA target, was detected in all germ cells in *Dazl*$^{-/-}$ embryonic testes whereas OCT4 expression was heterogeneous in *Dazl*$^{+/-}$ germ cells, suggesting the effect of DAZL on OCT4 expression, if any, is likely to be indirect (Figure 4B). Indeed, SOX2 has been shown to associate with the *Oct4* promoter and regulate *Oct4* expression (Chew et al., 2005). The expression of these pluripotency genes was also quantified by counting the number of positive cells in equal numbers of sections of *Dazl*$^{-/-}$ and *Dazl*$^{+/-}$ gonads as shown in Figure S5A.

We further explored SOX2 protein expression at an earlier developmental stage in E12.5 male gonads. Using confocal microscopy we quantified SOX2 protein in *Dazl*$^{-/-}$ PGCs and heterozygous controls, by normalizing the SOX2 signal to that of LIN28A in the same cells. We observed a 2-fold increase in the normalized SOX2 protein levels in the *Dazl*$^{-/-}$ PGCs compared to wild-type/heterozygotes, whereas, at the RNA level, *Sox2* transcript was upregulated 1.2-fold (Figure S5B). This indicates that already at E12.5 DAZL exerts its role, predominantly as a translational inhibitor of SOX2.

DAZL Suppresses Apoptosis in Developing PGCs

We also observed that the *Dazl*$^{-/-}$ germ cells in embryonic testes failed to enter mitotic arrest, as previously reported (Figures 4B, bottom panel, and S5A) (Gill et al., 2011). This, however, does not result in an increased number of germ cells; quite to the contrary, the majority of seminiferous tubules in *Dazl*$^{-/-}$ embryonic testes were empty,

because in the absence of *Dazl* expression, germ cells are lost to apoptosis beginning at E14.5 (Lin and Page 2005). Interestingly, in addition to mRNAs related to pluripotency, we observed that DAZL-IP also enriched for mRNAs of proapoptotic genes. *Caspase 2*, *7*, and *9* specifically coimmunoprecipitated with DAZL from in vitro PGC cultures and testicular lysates, whereas *Caspase 4* and *6*, which are also expressed in embryonic gonads, did not (Figure 5A). Analysis of the 3′ UTRs of these Caspases revealed that *Caspase 2*, *7*, and *9* mRNAs indeed contain archetypical DAZL binding sites, whereas DAZL-binding sites are rare or absent in other members of the Caspase family (Figure 5B). The interaction between DAZL and Caspase mRNAs suggests that DAZL may directly regulate apoptosis in PGCs by suppressing the translation of Caspase RNAs. *Caspase 7* is particularly intriguing, because this is a pivotal caspase in the execution stage of apoptosis. To explore the functional role of DAZL in regulating Caspase expression, we performed immunohistochemistry on E13.5 embryonic gonads of *Dazl*$^{-/-}$ mice and control heterozygous littermates. As shown in Figure 5C, CASPASE7 protein is abundantly expressed in PGCs of *Dazl*$^{-/-}$ gonads and it colocalizes with LIN28-positive germ cell clusters. In contrast, CASPASE7 was found only sporadically in control gonads, but it is absent from cells expressing the PGC marker LIN28 (Figure 5C). Together, these data demonstrate that CASPASE7 is a specific mRNA target of DAZL, and loss of DAZL expression releases CASPASE7 translational inhibition, thereby allowing PGCs to enter apoptosis.
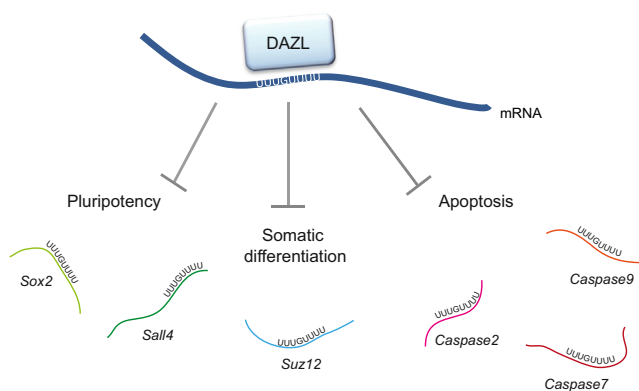
Figure 6. Schematic Representation of DAZL Regulation of Pluripotency, Somatic Differentiation, and Apoptosis

DISCUSSION

In this study, we utilized in vitro generation of PGC-like cells from ESCs and identified a network of DAZL-interacting proteins and mRNAs that plays a central role in controlling pluripotency, differentiation, and apoptosis. We demonstrate that the in vitro PGC-like cells recapitulate many aspects of in vivo PGC development, including the developmental timing of germ cell marker expression (*Stella* versus GFP). In vitro PGC-like cells are clearly distinct from the ESCs in terms of their gene expression profile as well as the subcellular localization of DAZL, which is homogeneously distributed in the cytoplasm in ESCs but localized to P-bodies in nascent PGCs, both in vitro and in vivo. Nonetheless, the in vitro-generated PGCs exist outside the context of the embryonic gonad and as such lack control by the stromal microenvironment, which may limit their in vitro development to stages beyond the PGC state. The lack of stromal control may also account for the fact that the in vitro PGC-like cells are developmentally more heterogeneous than their in vivo counterparts. We therefore call these cells PGC-like, to reflect this important difference with in vivo PGCs. Nonetheless, the in vitro generation of large numbers of PGC-like cells from ESCs provides an important tool to perform robust biochemical analysis on this otherwise inaccessible cell population.

Silencing of Key Pluripotency Factors
Contrary to previous reports, DAZL was found to target mRNAs for translational inhibition in embryonic gonads. Furthermore, the identities of proteins coimmunopurified with DAZL also indicated that DAZL has a more complex role in regulating its downstream mRNAs than as a recruiter for poly(A) binding proteins as previously believed.

The transition of PGCs into germ cells that is followed by sexual differentiation to the gametes (around E13.5) marks a key developmental milestone in germ cell development. We demonstrated that at this transition, DAZL is required for the downregulation of pluripotency genes *Sox2*, *Sall4*, and *Suz12*, the transcripts, which physically interact with DAZL. Our data reveal that in nascent PGCs, Dazl acts as a translational inhibitor. *Sox2* and *Sall4* are part of the core pluripotency transcriptional network required for embryonic stem cell self-renewal (Zhang et al., 2006), and therefore their inhibition would put PGCs at risk of somatic differentiation. Interestingly, *Suz12*, a core component of the polycomb repressor complex 2, is a DAZL target as well. *Suz12* has been shown to be essential for ESC differentiation. In the absence of the polycomb repressor complex, ESCs are stuck in a perpetual state of pluripotency (Pasini et al., 2004, 2007). Thus, DAZL-mediated silencing of both pluripotency factors and the polycomb complex allows PGCs to reduce the risk of teratoma formation by inhibition of the pluripotent program while simultaneously preventing somatic differentiation.

Silencing of Proapoptotic Factors
PGCs that fail to develop into oocytes and spermatogonia are the cause of germ cell tumors such as testicular teratomas in the adult animal. The requirement of DAZL in limiting pluripotency in PGCs implicates that the absence of DAZL may lead to an increase in teratoma incidences in mice. This is, however, not observed in the $Dazl^{-/-}$ mice in either C57Bl6 or mixed background. The lack of germ cell tumors in the C57Bl6 $Dazl^{-/-}$ mice is likely due to the drastic reduction in germ cell numbers starting at embryonic stages. Lin and Page showed that the loss of PGCs in the $Dazl^{-/-}$ embryo is due to increased apoptosis, indicating a role of DAZL in repressing programmed cell death in PGCs (Lin and Page 2005). Our finding that DAZL regulates the expression of key Caspases reveals an elegant fail-safe mechanism that prevents stray PGCs from forming teratomas by sensitizing them to apoptotic cell death. Although Caspase expression is required for cells to enter apoptosis, it is in itself not sufficient, because the inactive Caspase needs to be triggered by the apoptotic cascade to become active. We hypothesize that a ubiquitous proapoptotic signal may be presented to PGCs around E12.5/E13.5, but that this signal is only effective in cells in which translation of the proapoptotic proteins is released, providing a powerful selective mechanism to eliminate aberrant PGCs.

Conclusions
Based on these observations, we propose a model as shown in Figure 6, in which DAZL recruits and traps mRNAs encoding specific pluripotency factors to cytoplasmic granules for translational suppression. Inhibition of SOX2 and SALL4 translation affect the pluripotency network directly,

whereas translational inhibition of SUZ12 has a dual effect and suppresses somatic differentiation as well. In addition to its role as a suppressor of pluripotency and differentiation, DAZL is also a gatekeeper of apoptosis in PGCs. This incorporates an elegant fail-safe mechanism into the PGC system, in which the loss of pluripotency regulation simultaneously triggers germ cell death and prevents germ cell tumor formation.

EXPERIMENTAL PROCEDURES

Cell Culture and In Vitro PGC Differentiation

Mouse ES cells were derived and maintained in standard ES+Lif medium on γ-irradiated feeder MEFs. For in vitro PGC differentiation, mouse ES cells were grown to confluency, trypsinized, and resuspended in differentiation medium: modified IMDM (Invitrogen 12440-053) containing 15% heat inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamate (Invitrogen), 1 × penicillin/streptomycin (Invitrogen), 1 × nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.2 mg/ml holo-Transferrin (Sigma T1283), 0.1 mM 1-thioglycerol, 25 μg/ml ascorbic acid (Sigma A4403), and 2.5 μg/ml plasmocin (InVivoGen). The feeder MEFs were depleted by incubation on tissue culture plastic. The ES cells were then seeded onto gelatin-coated tissue culture plates at 2×10^5 cells/cm^2. Media was refreshed 2 days after seeding (D2) and then every day afterward. The cells were harvest by digestion with trypsin (0.25%, Invitrogen) and collagenase IV (0.25 mg/ml, Invitrogen). In both *Stella*-GFP and *Dazl*-GFP lines, GFP$^+$ cells are visible in culture at D5. In *Dazl*-GFP lines, GFP$^+$ cells can been seen as clusters on the ridges in the differentiation culture, which at this point is multilayer and highly heterogeneous.

Generation of Dazl-GFP ES Cell Lines and Transgenic Mouse

Construction of the targeting vector is described in Supplemental Experimental Procedures. The *Dazl*-GFP/Puro/Zeo BAC was linearized by PmeI digestion, purified by phenol/chloroform extraction, and electroporated into V6.5 mouse ES cells. The ES cells were selected in 2 μg/ml puromycin, and the resulting colonies were screened by Southern blotting. New *Dazl*-GFP ESC lines were also generated from transgenic blastocysts on 129/Bl6 mixed background using standard methods. The confirmed floxed clone prior to CRE excision was used to generate chimeric mice by blastocyst injection. The transgenic mice used in this study were on a mixed 129/Bl6 background. All animal experiments were approved by the IACUC committee and conform to regulatory standards.

Quantitative PCR Gene Expression Analysis

Total RNA was extracted with Trizol reagent (Invitrogen) and treated with DNA-free kit (Ambion), and first-strand cDNA was synthesized with SuperScript III reverse transcriptase using random primers (Invitrogen). Quantitative PCR (qPCR) was performed using SYBR Green master mix in a StepOne Plus cycler (Applied Biosciences). The primer sequences are listed in Supplemental Experimental Procedures.

Microarray Analysis

For genome-wide expression analysis, total RNA was extracted using Trizol reagent (Invitrogen) and labeled and hybridized to Agilent Whole Mouse Genome Oligo 4X44K Microarrays (one-color platform) according to the manufacturer's protocols. The gene expression results were analyzed using GeneSifter microarray analysis software, the DAVID bioinformatics tools, and MATLAB programs developed in house.

Fluorescence Activated Cell Sorting

ESCs, in vitro PGC-like cells, and in vivo PGCs from dissected gonads were sorted in fractions with GFP-positive and GFP-negative cells using a Becton Dickinson fluorescence-activated cell sorting (FACS) Aria cell sorter or analyzed on a Becton Dickinson FACS Calibur cell analyzer.

Immunofluorescence Microscopy

Murine gonads and testes were fixed in 4% paraformaldehyde (PFA), soaked in sucrose steps, embedded, and cryosectioned at 8–10 μm. Cells in suspension were cytospun onto Superfrost plus slides and fixed in 4% PFA. Tissue sections were treated with citrate buffer for antigen retrieval. The fixed cells and tissues were permeabilized in 0.1% Triton X-100/PBS and blocked in 1% BSA in 0.5% Triton X-100 in PBS for 30 min at room temperature (RT). The cells/tissue were then incubated in primary antibodies for 16 hr at 4°C, washed, and incubated with secondary antibodies for 30 min at RT, washed, and mounted in Vectashield with 1 ng/ml DAPI. Cells and tissues were imaged with the NIKON A1 confocal system. Fluorescence signals were quantitated using ImageJ tools, and statistical analyses were computed in GraphPad. The antibody sources are listed in Supplemental Experimental Procedures.

Immunoprecipitation of Protein-RNA Complexes

Two methods were used to coimmunoprecipitate RNA species interacting with *Dazl*-GFP-V5. For the UV-crosslinking method, 10 cm plates of 2D-differentiated cultures were washed twice in ice-cold PBS and UV irradiated twice at 400 mJ/cm^2 (Stratalinker 1500). Cells were lysed in 750 μl lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 0.5% Igepal) supplemented with 2 mM vanadyl ribonucleotide complex (NEB), 100 U/ml RNaseOUT (Invitrogen), 1 mM phenylmethanesulfonylfluoride, 1 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM DTT. Cell lysates were cleared by centrifugation, and 3 mg of total protein lysates was precleared and then incubated with Dynabeads protein A (Invitrogen) with anti-V5 antibody (Invitrogen), anti-GFP (Abcam ab6556), anti-PABP1 (Abcam ab21060), or normal mouse IgG at 4°C for 2–3 hr with rocking. The beads were then washed three times in PXL buffer (1 × PBS, 0.1% sodium dodecyl sulfate, 0.5% Igepal, 0.5% sodium deoxycholate) with all supplements, two times in high-salt PXL buffer (0.5 M NaCl) and one time in wash buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 0.05% Igepal) with supplements, resuspended in 100 μl wash buffer with supplements, and treated with proteinase K (20 μg per reaction, Invitrogen) at 37°C for 20 min. The coprecipitated RNA was isolated using the Trizol reagent (Invitrogen) following the manufacturer's protocols. For the noncrosslinking method, the cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM MgCl$_2$, 0.5% Igepal with

all supplements listed above. Immunoprecipitation was similarly performed, and the beads were washed five times in wash buffer with all supplements, 1 mM DTT, and all supplements. The RNA was extracted from the beads using Trizol reagent (Invitrogen).

Motif Identification from RNA-IP Data

DRIM (Eden et al., 2007) was used to discover motif in 3′ UTR sequences from RefSeq database. The fold change of each gene was used for ranking and background 3′ UTR sequences were randomly selected from RefSeq entries, with a ranking score 0. For parameters, "-rank" and "-RNA" were specified. As a control, we also randomized the ranking scores in the list (including both foreground and background sequences) and recomputed the motif. Motifs in individual mRNAs were scored and identified using SFmap (Paz et al., 2010).

ShRNA Knockdown

Target sequences for shRNA knockdown were selected using the pSicoOligomaker program (Dr. A. Ventura) and cloned into pLentiLox3.7 with a GFP marker or with a tdTomato marker (a gift from Dr. L. Daheron). The target sequences are listed in Supplemental Experimental Procedures.

Affinity Capture Protein Mass Spectrometry

Dazl-GFP and associated proteins were immunoprecipitated with anti-V5 antibody (Invitrogen) as described above from day 12 differentiated cells. Parental V6.5 cells in matched conditions were used as negative control. The immunoprecipitants were resolved by 2D gel electrophoresis following the manufacturer's protocols (ZOOM [pH 3-10] nonlinear strips, NuPAGE 4%–12% Bis-Tris ZOOM gel, Invitrogen), and the immunoprecipitated proteins were visualized by SyproRuby (Sigma) staining. Protein spots specific to Dazl-GFP cell lines were excised and identified by microcapillary LC/MS/MS analysis (Taplin Biological Mass Spectrometry Facility, Harvard Medical School). Immunoprecipitants or 25 µg of whole-cell lysates were used per sample in immunoblotting. The antibody sources are listed in Supplemental Experimental Procedures.

ACCESSION NUMBERS

All RNA-IP microarray data have been deposited at the NCBI Gene Expression Omnibus (GEO) under accession number GSE39547.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr. 2014.09.003.

AUTHOR CONTRIBUTIONS

H-H.C., M.W., and N.G. conceived work and wrote the manuscript. H.-H.C., M.W., and N.G. designed the experiments and analyzed the data. H.-H.C. and M.W. performed the majority of the experiments. D.B.B. helped with the analysis of the P-body complexes. J.M. and A.J.H. analyzed the protein interaction data. E.M and R.W. helped confirm the translational-suppressive effect of the Dazl complex. X.C. and C.T. assisted with the in vitro differentiation experiments. J.W. performed in silico analysis of DAZL binding sites. A.Y., Y.-F.C., and C.B. assisted with the generation of *Dazl*-GFP mouse. A.K. supervised the in silico analysis. N.G. supervised all aspects of the project.

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