

1-15-2007

## Intermolecular Interactions of Homologs of Germ Plasm Components in Mammalian Germ Cells

Mark S. Fox

*Center for Reproductive Sciences; Human Embryonic Stem Cell Research Center; Department of Obstetrics, Gynecology and Reproductive Sciences; Institute for Stem Cell and Tissue Biology; Departments of Physiology and Urology; University of California at San Francisco*

Amander T. Clark

*Center for Reproductive Sciences; Human Embryonic Stem Cell Research Center; Department of Obstetrics, Gynecology and Reproductive Sciences; Institute for Stem Cell and Tissue Biology; Departments of Physiology and Urology; University of California at San Francisco*

Mohammed El Majdoubi

*Center for Reproductive Sciences; Human Embryonic Stem Cell Research Center; Department of Obstetrics, Gynecology and Reproductive Sciences; Institute for Stem Cell and Tissue Biology; Departments of Physiology and Urology; University of California at San Francisco, mohammed.elmajdoubi@dominican.edu*

### Survey: let us know how this paper benefits you.

Jean-Louis Vigne

*Center for Reproductive Sciences; Human Embryonic Stem Cell Research Center; Department of Obstetrics, Gynecology and Reproductive Sciences; Institute for Stem Cell and Tissue Biology; Departments of Physiology and Urology; University of California at San Francisco*

### Recommended Citation

Fox, Mark S.; Clark, Amander T.; El Majdoubi, Mohammed; Vigne, Jean-Louis; Urano, Jun; Hostetter, Chris E.; Griswold, Michael D.; Weiner, Richard I.; and Reijo Pera, Renee A., Intermolecular Interactions of Homologs of Germ Plasm Components in Mammalian Germ Cells (2007), *Collected Faculty and Staff Scholarship*, 208. <https://doi.org/10.1016/j.ydbio.2006.08.047>

See next page for additional authors

<http://dx.doi.org/https://doi.org/10.1016/j.ydbio.2006.08.047>

<https://doi.org/10.1016/j.ydbio.2006.08.047>

This Article is brought to you for free and open access by the Faculty and Staff Scholarship at Dominican Scholar. It has been accepted for inclusion in Collected Faculty and Staff Scholarship by an authorized administrator of Dominican Scholar. For more information, please contact [michael.pujals@dominican.edu](mailto:michael.pujals@dominican.edu).

---

**Authors**

Mark S. Fox, Amander T. Clark, Mohammed El Majdoubi, Jean-Louis Vigne, Jun Urano, Chris E. Hostetler, Michael D. Griswold, Richard I. Weiner, and Renee A. Reijo Pera

Published in final edited form as:

*Dev Biol.* 2007 January 15; 301(2): 417–431. doi:10.1016/j.ydbio.2006.08.047.

## Intermolecular Interactions of Homologs of Germ Plasm Components in Mammalian Germ Cells

Mark S. Fox<sup>a</sup>, Amander T. Clark<sup>a</sup>, Mohammed El Majdoubi<sup>a</sup>, Jean-Louis Vigne<sup>a</sup>, Jun Urano<sup>a</sup>, Chris E. Hostetler<sup>b</sup>, Michael D. Griswold<sup>b</sup>, Richard I. Weiner<sup>a</sup>, and Renee A. Reijo Pera<sup>a,\*</sup>

<sup>a</sup>Center for Reproductive Sciences; Human Embryonic Stem Cell Research Center; Department of Obstetrics, Gynecology and Reproductive Sciences; Institute for Stem Cell and Tissue Biology; Departments of Physiology and Urology; University of California at San Francisco; San Francisco, CA 94143-0556, USA

<sup>b</sup>School of Molecular Biosciences, Washington State University; Pullman, WA 99163-3520, USA

### Abstract

In some species such as flies, worms, frogs, and fish the key to forming and maintaining early germ cell populations is the assembly of germ plasm, microscopically-distinct egg cytoplasm that is rich in RNAs, RNA-binding proteins and ribosomes. Cells which inherit germ plasm are destined for the germ cell lineage. In contrast, in mammals, germ cells are formed and maintained later in development as a result of inductive signaling from one embryonic cell type to another. Research advances, using complementary approaches, including identification of key signaling factors that act during the initial stages of germ cell development, differentiation of germ cells *in vitro* from mouse and human embryonic stem cells and the demonstration, that homologs of germ plasm components are conserved in mammals, have shed light on key elements in the early development of mammalian germ cells. Here, we use FRET (Fluorescence Resonance Energy Transfer) to demonstrate that living mammalian germ cells possess specific RNA/protein complexes that contain germ plasm homologs, beginning in the earliest stages of development examined. Moreover, we demonstrate that although both human and mouse germ cells and embryonic stem cells express the same proteins, germ cell specific protein/protein interactions distinguish germ cells from precursor embryonic stem cells *in vitro*; interactions also determine sub-cellular localization of complex components. Finally, we suggest that assembly of similar protein complexes may be central to differentiation of diverse cell lineages and provide useful diagnostic tools for isolation of specific cell types from the assorted types differentiated from embryonic stem cells.

### Keywords

germ cells; germ cell development; DAZ; DAZL; PUM2; PUM1; NANOS; germ plasm; RNA-binding proteins

---

\*Corresponding author. Email: reijoperar@obgyn.ucsf.edu

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## Introduction

In most organisms, germ cells are set aside from the somatic cells that form the rest of the organism, early in embryonic development (reviewed by Ikenishi, 1998; Wylie, 2000; Santos and Lehmann, 2004; Zhou and King, 2004). Elegant studies dating from the early- to mid-20<sup>th</sup> century have demonstrated that in organisms such as flies, worms, frogs and fish, the germ cell lineage is established by the assembly of microscopically-detectable germ plasm present in the oocyte and the subsequent segregation of germ plasm to cells which are destined to be germ cells (Ikenishi, 1998; Wylie, 2000; Santos and Lehmann, 2004; Zhou and King, 2004). In contrast, in mammals, it is clear that microscopically-distinct germ plasm is not assembled in the oocyte and that germ cells are set aside later in development by inductive signaling from extra-embryonic cells to pluripotent precursors in the proximal epiblast (Wylie, 2000; Santos and Lehmann, 2004). Yet, recently, it has been shown that many genes that encode homologs of germ plasm components are conserved in mammals, including genes such as *DAZ* (*Deleted in AZoospermia*), *DAZL* (*DAZ-Like*), *Nanos*, *Pumilio*, and *Vasa* homologs (Reijo et al., 1995; Lin and Spradling, 1997; Forbes and Lehmann, 1998; Parisi and Lin, 1999; Castrillon et al., 2000; Tanaka et al., 2000; Mochizuki et al., 2001; Jaruzelska et al., 2003; Moore et al., 2003; Tsuda et al., 2003). Indeed, where functional data is available, these genes are required for establishing, maintaining and differentiating germ cell populations (Reijo et al., 1995; Eberhart et al., 1996; Reijo et al., 1996; Ruggiu et al., 1997; Maegawa et al., 1999; Houston and King, 2000; Karashima et al., 2000; Tsuda et al., 2003; Tung, 2006; Lin and Page, 2005). For example, in humans, deletions and variants of *DAZ* homologs are associated with the production of very few or no germ cells (Reijo et al., 1995; Reijo et al., 1996), whereas, in *Xenopus*, *xDazl* encodes a component of germ plasm that is required for primordial germ cell development initially and subsequent development of mature germ cell types (Houston and King, 2000; Padmanabhan and Richter, 2006). In other organisms, such as zebrafish and salamanders, it has also been shown that *DAZL* homologs encode germ plasm homologs (Howley and Ho, 2000; Johnson AD, 2001). In addition, the localization and conservation of germ plasm components such as *Pumilio* and *Nanos* homologs across diverse species is also well-documented (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Asaoka-Taguchi et al., 1999; Parisi and Lin, 1999; Subramaniam and Seydoux, 1999; Kopranner et al., 2001; Nakahata et al., 2001; Jaruzelska et al., 2003; Tsuda et al., 2003; D'Agostino et al., 2006).

Recent studies demonstrated that mouse embryonic stem cells (mESCs) are capable of differentiating into female and male germ cells *in vitro* (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Nayernia et al., 2006). Hubner and colleagues noted that oocyte differentiation from mESCs was obtained via spontaneous differentiation of adherent cultures, as indicated by expression of genes such as *Vasa*, *Gdf9* and *Scp3*, and corroborated by morphological evidence and production of follicular steroids (Hubner et al., 2003). Two other studies reported the differentiation of spermatogenic cells from mESCs (Toyooka et al., 2003; Geijsen et al., 2004). Toyooka and colleagues differentiated mESCs to embryoid bodies and analyzed expression of germ cell-specific markers including a reporter GFP integrated into the mouse *Vasa* locus (Toyooka et al., 2003). Initial differentiation *in vitro* was then followed by transplantation studies, in which the authors observed that transplanted primordial germ cells readily formed sperm, whereas, transplantation of undifferentiated mESCs resulted in teratoma formation (Toyooka et al., 2003). Geijsen and colleagues extended these studies with analysis of imprinting and further evidence that haploid male gametes form *in vitro* and are capable of promoting development to blastocyst stage, when injected into oocytes (Geijsen et al., 2004). Finally, most recently, another group demonstrated that mESC-derived male gametes can generate offspring in mice, thus bringing the work full circle to the ultimate proof of functional gametogenesis *in vitro* (Nayernia et al., 2006).

Concurrent with studies in mice, human embryonic stem cells (hESCs) were shown to differentiate to germ cells (Clark et al., 2004a; Clark et al., 2004b). Three independently-derived hESC lines were differentiated to embryoid bodies and assayed for germ cell development *in vitro* (Clark et al., 2004a; Clark et al., 2004b). Markers examined included those that were used to assay mouse germ cell differentiation *in vitro* as well as others diagnostic of different stages of germ cell development. It was shown that the earliest steps of human germ cell development, including expression of VASA and meiotic synaptonemal components, occurred *in vitro* (Clark et al., 2004a; Clark et al., 2004b). A caveat, however, in these studies in both mice and humans, is the common expression of protein and mRNA markers in both primordial germ cells and ESCs. This observation has in fact led to the hypothesis that hESCs are closely related, or even identical, to early germ cell precursors (Clark et al., 2004a; Clark et al., 2004b; Zwaka and Thomson, 2005).

Here we sought to address the hypothesis that specific interactions of mammalian homologs of germ plasm components accompany formation and/or maintenance of early germ cell populations, in a process analogous to the assembly of germ plasm in lower organisms. Thus, we tracked the intermolecular interactions, in living germ cells and embryonic stem cells, of two proteins that are homologs of germ plasm components: DAZL and PUM2. Mouse, human and frog homologs of these proteins, and other germ plasm homologs, were previously shown to interact specifically *in vitro* (Moore et al., 2003; Fox et al., 2005; Urano et al., 2005; Padmanabhan and Richter, 2006).

## Materials and Methods

### Preparation of Protein Homogenates of Mouse Testis

Murine testis were dissected and homogenized with a Dounce homogenizer in buffer containing 60mM KCl, 150mM or 500mM NaCl, 15mM HEPES, pH 7.8, 0.3M Sucrose, 14mM  $\beta$ -mercaptoethanol, Complete (Protease inhibitor cocktail; Roche Diagnostics Corporation) and 300  $\mu$ g/ml of RNase A (Sigma-Aldrich) or RNasin (Promega) as per manufacturer's instructions. Extract treated with RNase A or RNasin were incubated at room temperature for 5 min (Tsui et al., 2000b). The crude tissue homogenate was then centrifuged at  $3000 \times g$ , 5 min to remove large tissue debris and supernatant was collected.

### Size-Exclusion Chromatography

Murine testis and ESC extracts were fractionated by size exclusion chromatography on a 30ml S-300-HR column (Sigma-Aldrich) by low-pressure chromatography (Biologic LP system; BioRad). The column was equilibrated in 10 bed volumes of either physiological salt (150mM NaCl, 16mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 4mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 4mM KCl, pH 7.2) or high salt buffer (500mM NaCl, 16mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 4mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 4mM KCl, pH 7.2) prior to sample loading. Gel Filtration Standards (BioRad) were used to approximate the column resolution. Prior to loading, the supernatant was centrifuged at  $100,000 \times g$  for 1 hr; the sample was concentrated using Ultrafree Centrifugal Filter 30,000 (Millipore). Three mls of extract was loaded onto the column and collected at a flow rate of one 500 $\mu$ l fraction per minute; protein peaks were detected by UV monitoring at 280nm.

### Immunoblotting

Western blotting was essentially as described (Moore et al., 2003). Antisera dilutions were: DAZL (1:700), BOULE (1:500), PUM2 (1:500), NANOS1 (1:1000), VASA (1:500), DZIP1 (1:500), DAZAP1 (1:500; from Dr. P Yen, University of California Los Angeles), and Ribosomal P (1:500; Immovision). HRP-conjugated secondary antibodies were used as per manufacturer's instructions (Amersham Biosciences, Inc and Calbiochem). Western blots were visualized by chemiluminescence as described (Amersham Biosciences).

## GFP-Fusion Proteins

cDNAs for DAZL, and PUM2 were amplified by polymerase chain reaction (PCR) with primers carrying restriction enzymes *SpeI* at the 5' end and *EcoRI* at the 3' end and were then cloned into the Litmus 29 vector (New England Biolabs) (Urano et al., 2005). Each gene was inserted into the CMV promoter:peGFP-C1 vector (BD Biosciences) by digesting the cloned cDNAs with *NdeI*, creating blunt ends and then cutting with *XhoI* prior to ligating into the peGFP-C1 vector.

## RFP-Fusion Proteins

A monomeric red vector was constructed for the generation of RFP fusion constructs. pDsRed2-C1 vector (BD Biosciences) was digested with *AgeI* and *XhoI* and the tetrameric red protein coding sequence was removed. Primers were annealed and ligated into the remaining vector: 5'-ccggtaccatggagcagaaactcatctctgaagaggatctgggtggagatctactagtc-3' and 5'-tcgagactagtagatctccaccagatcctcttcagagatgagtttctgctccatgga-3' to introduce a MYC tag downstream of the CMV promoter. The coding region of the monomeric red fluorescent protein was PCR amplified with primers carrying restriction enzyme *Bgl II* at either end using the pRSETB plasmid as a template (a gift from R. Tsien, University of California at San Diego) (Campbell et al., 2002) and inserted into the modified vector using the *Bgl II* site located in the multi-cloning site of the modified vector (pnmRFP). pnmRFP fusion constructs were generated by digesting the Litmus 29 constructs with *SpeI* and *EcoRI* and inserting DAZL, and PUM2 at the 3' end of the RFP using the unique *SpeI* and *EcoRI* sites in the modified vector.

## Cell Culture, Transfection and Generation of Primary Germ Cell Cultures

The S4 spermatogonial cell line (M. Dym, Georgetown University) was routinely cultured as outlined at 33°C and 5% CO<sub>2</sub> (Feng et al., 2002). Two days before FRET experiments, mouse spermatogonial stem cells were seeded onto glass slides coated with 1:6 dilution of Matrigel (BD Biosciences) in DMEM/F-12 media described by (Feng et al., 2002). Cells were transfected with 500ng of each construct using Effectene transfection reagent according to manufacturer's instructions (Qiagen) 24 hours before FRET experiments. On the day of the experiment, Effectene-containing media was replaced with fresh media prior to analysis.

Mouse embryonic stem cells used in the current analysis were feeder-independent 129/Ola cells (from Dr. Nigel Killen, University of California at San Francisco). Mouse embryonic stem cells were maintained in a High Glucose DMEM media (Gibco), containing 10% FBS (Hyclone), 1× Nonessential Amino Acids (Gibco), 1mM L-glutamine (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 10<sup>6</sup> units/L ESGRO (Chemicon International). Two days before FRET analysis, mouse embryonic stem cells were seeded onto glass slides coated with 0.1% gelatin (Sigma-Aldrich) in High Glucose DMEM media described above. Cells were transfected with 2μg of each construct using TransFectin Lipid reagent according to manufacturer's instructions (Biorad), 24 hours before analysis. On the day of FRET analysis, media was replaced with fresh High Glucose DMEM media.

Human embryonic stem cell line, H9 (NIH code: WAO9; <http://stemcells.nih.gov/>) was cultured on irradiated CF1 mouse embryonic feeders (MEFs) plated at a density of 11,000-21,000/cm<sup>2</sup> in DMEM high glucose (Gibco, Inc) containing 10% Fetal Bovine Serum (Hyclone), 1× Penicillin/Streptomycin (Gibco) and 1× L-Glutamine (Gibco). Human embryonic stem cells were cultured in KSR media composed of Knockout DMEM (Gibco), containing 20% Knockout Serum Replacement (Gibco), 1× Nonessential Amino Acids (Gibco), 1mM L-glutamine (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4ng/mL bFGF (R&D Systems). Human embryonic stem cells were routinely passaged by incubation with Collagenase typeIV (Gibco), in Knockout DMEM (Gibco) and manual scrapping of



colonies. For FRET analysis, cells required feeder-free growth in the presence of matrigel. For this, three days prior to FRET experiments, irradiated CF1 mouse embryonic fibroblasts were plated at a density of 55,000-66,000 cells/cm<sup>2</sup> in CFI media. The following day, CF1 media was replaced with KSR media containing 4ng/mL bFGF; the KSR media was conditioned by the CF1 feeders for 24 hours at 37°C in 5% CO<sub>2</sub>, before collection, addition of an additional 8ng/mL bFGF and filtering through a 0.2µm filter. On the day of conditioned media collection, human embryonic stem cells were treated with Collagenase type IV, re-suspended in filtered KSR conditioned media and seeded onto glass slides that had previously been coated with a 1:15 dilution of Matrigel (BD Biosciences). Human embryonic stem cells were cultured for 24 hours on matrigel before replacing the media with fresh conditioned KSR media containing 8ng/mL bFGF. Human embryonic stem cells were transfected with 3µg of each construct using Effectene (Qiagen) according to manufacturer's instructions. The following day, the Effectene-containing media was removed and replaced with fresh, conditioned KSR media with bFGF.

Male mouse embryonic germ cell (mEGC) lines generated at 8.5 dpc (Onyango et al., 2002) and 12.5 dpc (Labosky et al., 1994) were used at passage 8 and 22, respectively. mEGCs were cultured on irradiated STO feeders plated at 11,000-21,000/cm<sup>2</sup> in DMEM high glucose (Gibco) containing 10% Fetal Bovine Serum (Hyclone), 1x Penicillin/Streptomycin (Gibco) and 0.5 × L-Glutamine (Gibco). mEGCs were cultured in EG culture medium with ESGRO (1000 Units/ml) composed of High Glucose DMEM media (Gibco), containing 15% FBS (Hyclone), 1x Penicillin/Streptomycin (Gibco), 1 × L-Glutamine (Gibco), 1× Nonessential Amino Acids (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 10<sup>6</sup> units/L ESGRO (Chemicon International). Three days prior to FRET, 55,000-66,000 STO feeder cells/cm<sup>2</sup> were plated onto glass slides coated with a 1:15 dilution of Matrigel (BD Biosciences). The following day, STO media was removed and replaced with EG media with ESGRO and mEGCs were seeded onto the feeders and transfected with 3µg of each construct using TransFectin Lipid reagent (Biorad) according to manufacturer's instructions. The following day, the media was removed and replaced with fresh EG media containing ESGRO.

Mouse male gonocytes from day 3 mice were isolated from testes that were decapsulated and resuspended in Hanks media (Gibco) with Trypsin (0.025 mg/ml; Sigma-Aldrich) and DNase (5 µg/ml; Sigma-Aldrich) and then incubated at 37 °C for 30 min. Trypsinization was stopped by addition of 1ml trypsin inhibitor (Sigma-Aldrich) and the resulting suspension was centrifuged. The cell pellet was resuspended in Hanks media containing collagenase (1 mg/ml; Sigma-Aldrich), and DNase (5 µg/ml) for 30 min at 37 °C with gentle agitation every 10 mins. Dispersed cells were centrifuged and resuspended in 0.5% bovine serum albumin (BSA fraction V; Sigma-Aldrich) in Hanks medium. Cells from the dissociated tubules were separated by sedimentation velocity at unit gravity at 4°C using a 2-4% (w/v) BSA gradient in Hanks medium. Essentially, cells were bottom-loaded into a STAPUT chamber as previously outlined in 30 ml of Hanks media containing 0.5% BSA, and a gradient was simultaneously generated using 175 ml of each of two media, one supplemented with 2% BSA and the other with 4% BSA (McLean et al., 2003; Kubota et al., 2004). Cells were allowed to sediment for 3 h and fractions of 3 ml were collected from the bottom of the gradient. Fractions enriched for spermatogonial stem cells were identified by microscopy, pooled and pelleted. Pooled fractions were resuspend in PBS with 0.1% BSA and incubated with anti-mouse Thy1 antibody (BD Biosciences), 20 min on ice.. Cells were then washed in PBS plus 0.1% BSA prior to incubation on ice with M-450 Sheep anti-Rat Dynabeads (DynaL Biotech ASA) for another 20 min. Gonocytes were further purified by magnetic activated cell sorting (MACS) by placing the cell bead suspension in a Magnetic Particle Concentrator (DynaL Biotech ASA). The cell suspension was washed 4 times in PBS 0.1% BSA buffer prior to resuspension in serum free medium and plating on irradiated STO feeders as described (Kubota et al., 2004). Prior to FRET, 55,000-66,000 STO feeder cells/cm<sup>2</sup> were plated onto glass slides that were coated with 0.1% gelatin. The following day, STO media was removed and replaced with serum free

medium and gonocytes were seeded onto the STO feeders. For transfection, gonocytes were transfected with 3 $\mu$ g of each construct using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The following day, the Lipofectamine containing media was removed and replaced with fresh serum free media before analysis.

Mouse Leydig MA-10 cells (Ascoli, 1981) were maintained in Waymouth's complete medium MB752/1 (Invitrogen) containing 20mm HEPES, 1.2 g/liter sodium bicarbonate, 15% heat inactivated donor horse serum and 1x Penicillin/Streptomycin (Gibco). Two days before FRET analysis, MA-10 cells were seeded onto glass slides coated with 0.1% Matrigel (BD Biosciences) diluted in Waymouth's complete medium described above. Cells were transfected with 1 $\mu$ g of each GFP-fusion construct and 2 $\mu$ g of each RFP-fusion construct using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions, 24 hours before analysis. The following day, the Lipofectamine containing media was removed and replaced with fresh Waymouth's complete medium before analysis.

HEK293 cells were maintained in DMEM medium (Invitrogen) containing 10% fetal bovine serum and 1x Penicillin/Streptomycin (Gibco). Two days before FRET analysis, HEK293 cells were seeded onto glass slides coated with 0.1% Matrigel (BD Biosciences) diluted in DMEM medium described above. Cells were transfected with 2 $\mu$ g of each construct using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions, 24 hours before analysis. The following day, the Lipofectamine containing media was removed and replaced with fresh DMEM medium before analysis.

### Live Cell Image Collection and Image Analysis

FRET allows the visualization of protein interactions in live cells (Day and Schaufele, 2005). Cell-coated coverslips were placed in a temperature-controlled (37°C), modified Sykes-Moore Chamber mounted on a Nikon TE2000 inverted fluorescence microscope. Cells were perfused with DMEM/F-12 medium (without phenol red) with 0.5% Fetal Bovine Serum (Invitrogen) and 2mM HEPES at a flow rate of 50 $\mu$ l/min. Cells were imaged under a 100X epifluorescence objective using a xenon light source (Lambda LS, Sutter Instrument Company). Images were collected sequentially through RFP (acceptor), eGFP (donor) and FRET filter channels as follows: RFP (excitation 500-560nm/emission 580-630), eGFP (excitation 480-495nm/emission filter 500-530 nm), FRET (excitation 480-495nm/emission 580-630). Corrected FRET was calculated for the entire image on a pixel-by-pixel basis using Metamorph software (Universal Imaging) after images were background-subtracted and corrected for bleed through values of red from eGFP (14%), green from RFP (1%), FRET from RFP (9%) and FRET from eGFP (27%) as described earlier (Weatherman et al., 2002). Corrected FRET was then normalized to the amount of Donor (FRET/eGFP) and plotted against the amount of acceptor relative to donor (RFP/eGFP). Data was generated from a minimum of 20 cells per experiment.

To further examine nuclear localization, we also built three dimensional images from optical slices. For this purpose, the spermatogonial cell line, S4, was cultured and transfected as described above. Nuclei of the cells were visualized using Hoechst 34580 (Invitrogen), by adding 10 $\mu$ l of a 10mg/ml stock to each well, 10 minutes prior to visualization. Image acquisition was operated under software control (METAMORPH, Universal Imaging, Downingtown, PA). For movie imaging, image stacks (x, y, and z planes) were acquired at two wavelengths (green, red or blue) for a single frame as follows: sequential planes were acquired at axial (z) to form a z-stack for two colors. Data sets were flattened along the z axis as maximum intensity projections, the images were overlaid and reconstituted as a three-dimensional image.



## Yeast Three-Hybrid Experiments

Yeast three-hybrid experiments and assays were as described (Moore et al., 2003; Urano et al., 2005). Essentially, to assay for PUM2 and DAZL interactions with the sequence of interest, cDNA was cloned into the pACT2 vector and interactions were assayed via filter assays as also described (Moore et al., 2003; Urano et al., 2005). The yeast strain L40c and plasmid pIII/MS2-2 were gifts from Dr M Wickens (University of Wisconsin, Madison). Each assay was replicated in three independent experiments. Specificity of the binding of PUM2 and DAZL to the *SDAD1* 3' UTR sequences was previously demonstrated (Fox et al., 2005).

## Results

### Biochemical Evidence for Distinct Complexes Containing the DAZL and PUM2 Proteins

To examine whether DAZL and PUM2 associate in one or more complexes that are diagnostic of embryonic stem cells and germ cells, we used size exclusion chromatography to analyze the molecular mass of complexes that contain these proteins. For this purpose, cytoplasmic extracts were prepared from mouse testis treated with either RNasin (to maintain RNA integrity) or RNase A (to destroy endogenous RNA) and proteins were separated on a size exclusion column in physiological and 500mM NaCl buffers. These experiments are designed to detect whether complexes are maintained primarily via RNA-dependent or independent mechanisms. We expect that if complexes are primarily dependent on binding to RNA, then RNase treatment should largely release the proteins from the complex. In contrast, if complexes are primarily dependent on protein:protein interactions, then RNase treatment should have little effect but treatment with high salt buffers may disrupt protein:protein interactions. Our experimental design is as diagrammed (Fig. 1) and attempts to clarify whether one or several complexes were dependent or independent of RNA-protein or protein-protein interactions.

Elution profiles of both the RNasin- and RNase-treated extracts in physiological conditions resulted in three peaks at greater than 650 KD (KiloDaltons), approximately 44 KD and 1.35 KD (Fig. 2A; Panels I, II). Although both extracts showed similar profiles, the samples with intact RNA had a greater abundance of large complexes than those in which endogenous RNA was destroyed, demonstrating that some components likely associated in an RNA-dependent manner (Fig. 2A; Panel II).

Western blot analysis with antisera specific to DAZL and PUM2 demonstrated that both proteins were eluted in a high molecular weight peak of 650 KD or more to 158 KD, as calculated from elution peaks of protein size standards (Fig. 2B; Panel I). Notably, the molecular weight of DAZL and PUM2 monomers is 33 and 98 KD, respectively. Thus, both proteins were present predominantly in complexes that even exceed the molecular weight of homodimers that they readily form (Ruggiu and Cooke, 2000; Jaruzelska et al., 2003; Urano et al., 2005). Moreover, other proteins previously shown to interact with PUM2 and/or DAZL were also present in the higher molecular weight complexes including NANOS1, VASA (also termed Mvh (Mouse Vasa Homolog)), DZIP1 (DAZ-Interacting Protein-1), BOULE, and DAZAP1 (DAZ-Associated Protein-1) proteins. As a control, we also probed for the presence of the ribosomal protein, Ribosomal P, which was present in the complex and was dissociated with RNase A treatment, as expected (Fig. 2B; Panel I). RNase treatment also released the BOULE and DAZAP1 proteins from the complexes (Fig. 2B; Panel II). In contrast, the destruction of endogenous RNA did not result in significant changes in the distributions of DAZL, PUM2, NANOS1, VASA or DZIP1 proteins (Fig. 2B; Panel II). Antisera specific to PUM1 are not available, but given that PUM2 and PUM1 share more than 97% identity in the 150 amino-acid region required for interaction with DAZL (Moore et al., 2003), we expect that these proteins could interact in DAZL-containing complexes interchangeably.

We also tested whether the elution of DAZL, PUM2, NANOS1, VASA, DZIP1, BOULE, and DAZAP1 in protein complexes was susceptible to increased ionic strength that might affect both protein-protein and protein-RNA interactions (Fig. 2C). We observed that the profiles of protein elution differed significantly in high salt buffers relative to physiological salt conditions regardless of RNA status (Fig. 2C; Panels I and II). Nonetheless, the most dramatic differences were observed in samples with both high salt concentrations and RNase A. The large complex of proteins observed at more than 650 KD was nearly absent under these conditions and proteins were redistributed to fractions corresponding to lower molecular weights (Fig. 2C; Panel II).

When we used Western blotting to compare the relative distribution of DAZL, PUM2, NANOS1, VASA, DZIP1, BOULE, DAZAP1, and Ribosomal P proteins between extracts prepared in high and physiological salt conditions, we observed distinct differences. With an increase in salt concentration, DAZL protein predominately found in fractions of a mass corresponding to monomers and dimers (Fig. 2D; Panel I), a profile that was unaffected by the presence of RNase A (Fig. 2D; Panel II). Elution profiles for PUM2, DZIP1, and DAZAP1 were similarly affected by increased salt concentrations though not by the presence of RNase A. Changes in the profiles of PUM2 and DZIP1 were least dramatic with a limited shift to lower molecular weight fractions. Finally, NANOS1, VASA and BOULE appeared to be completely released in high salt and RNase treatment (Fig. 2D; Panels I and II). Thus, these data indicate that a combination of high salt and RNAase treatment largely destabilized many of the protein-protein and RNA-protein interactions.

### FRET Analysis of Intracellular Localization and Protein Interactions in the S4 Spermatogonial Stem Cell Line

Next, we used fluorescence energy resonance transfer (FRET) to track the interaction of the proteins DAZL and PUM2 in embryonic and germ line stem cells and to directly measure intermolecular interactions in living cells. For this purpose, we constructed DAZL and PUM2 fusion proteins with green fluorescent protein (eGFP) and monomeric red fluorescent protein (RFP). When eGFP and RFP proteins are less than 80 angstroms apart, excitation of eGFP should trigger the fluorescent emission of the RFP and the transfer of energy from donor to acceptor fluorophors should be detected as FRET.

We first focused on examining homodimerization of PUM2 and DAZL protein in the mouse spermatogonial stem cell line, S4. Fluorescence microscopy analysis of cells transfected with eGFP-DAZL and RFP-DAZL indicated that both fusion proteins were perfectly co-localized throughout the cells, in both the nucleus and cytoplasm (Fig. 3A - B; Panels I - IV). In addition, examination of the molecular interactions between eGFP-DAZL with RFP-DAZL indicated a strong FRET signal between eGFP-DAZL and RFP-DAZL that correlated precisely with colocalization of the molecules (Fig. 3B; Panels IV and V). To correct for variation in transfection efficiency and expression, the FRET/eGFP ratio was graphed against the relative amounts of eGFP and RFP fluorescence for each cell image collected. This indicated that the FRET/eGFP ratio increased linearly with the RFP/eGFP at a slope characteristic of the efficiency of energy transfer from eGFP to RFP (Fig. 3B; Panel VI).

This finding indicated that there was a direct interaction between the DAZL fusion proteins in living cells and confirmed that homodimerization occurs *in vivo* and can readily be detected. Similarly, it has been observed that PUM2 forms dimers, at least *in vitro* (Jaruzelska et al., 2003; Urano et al., 2005). In similar experiments to those described for DAZL protein, we observed that expression of both eGFP-PUM2 and RFP-PUM2 resulted in polypeptides that co-localized to the cytoplasm of the spermatogonia, in distinct, punctate structures, with little or no nuclear signal (Fig. 3C; Panels I - III). Moreover, FRET was clearly detected with the PUM2 fusion proteins (Fig. 3C; Panel V), and the FRET/eGFP ratio increased with the RFP/

GFP ratio in the cells confirming that the formation of PUM2 dimers occurs *in vivo* in spermatogonial stem cells (Fig. 3C-VI).

Since DAZL and PUM2 proteins formed homodimers that we could readily detect via FRET, we next analyzed molecular interactions between DAZL and PUM2. Since we only observed PUM2 expression in cytoplasm, we assumed that interaction between DAZL and PUM2 would only be seen in this intracellular compartment. However, in mouse spermatogonial stem cells transfected with DAZL-eGFP and RFP-PUM2, we observed that both PUM2 and DAZL colocalized throughout the cell, including within the nucleus (Fig. 3D; Panels I - III). Moreover, we observed clear FRET with FRET/donor ratios that increased with increasing acceptor/donor ratios (Fig. 3D; Panels V - VI); the interactions were dependent on the presence of DAZL and PUM2 as eGFP and RFP alone do not interact (Fig. 3E; Panels I - VI). Thus, these results indicated that DAZL and PUM2 form both homodimers and heterodimers in living germ cells, and that hetero-dimerization presumably in the cytoplasm resulted in migration of the complexes to the nucleus or their retention in that compartment. Migration of PUM2 into the nucleus of the cells, in the presence of DAZL, was substantiated by staining the nuclei of the cells with Hoechst vital dye and examining co-localization of the nuclei and expressed fusion proteins (Fig. S1). In addition, we examined the localization of PUM2 and DAZL by constructing three dimensional images from optical slices in the x, y and z axis. Reconstruction of the cell allows one to visualize the cell from different angles and demonstrated the localization of the proteins to the nucleus (Supplemental Data; Movies 1-4).

A curiosity of these experiments is the observation that the low levels of endogenous DAZL protein do not appear to transport the bulk of the PUM2 fusion protein to the nucleus; nonetheless, we suspect that there are low levels of PUM2 transported via endogenous DAZL but that due to limitations of this technology, we are unable to detect the movement above background (Supplemental Data; Movie 4). This is most likely due to the high expression of the fusion proteins and the limited amount of endogenous proteins, not enough fluorescent protein is transported to be detected by the imaging software.

### **FRET Analysis of Intracellular Localization and Protein Interactions in Primary Cultures of Germ Cells from 3 Day Old Mice**

The spermatogonial stem cell line, S4, was established from undifferentiated spermatogonia from 6-day old BALB/C mice after immortalization with mouse telomerase (Feng et al., 2002). To address whether protein interactions detected by FRET also occur in primary germ cells, we cultured primary germ cells from 3 day old mice and co-transfected them with eGFP-DAZL and RFP-DAZL constructs (Fig. 3F; Panels I - II). As in the immortalized germ cell line, both proteins co-localized throughout the primary germ cells and homodimerization was readily detected (Fig. 3F; Panel III-V). The FRET signal was confirmed by observation of FRET/donor ratios that increased with increasing acceptor/donor ratios (Figs. 3F; Panel VI). Similar analysis with eGFP-PUM2 and RFP-PUM2 demonstrated co-localization in the cytoplasm of the primary germ cells in distinct structures and a clear FRET signal was detected (Fig. 3G; Panels I - VI). Moreover, DAZL and PUM2 formed heterodimers both in the cytoplasm and nucleus as in the S4 spermatogonial stem cell line (Fig. 3H; Panels I - VI). Taken together, these results indicated that formation of homo- and hetero-dimers of DAZL and PUM2 proteins occurred in both immortalized and primary cultures of premeiotic mouse germ cells. In addition, localization of PUM2 in the nucleus was dependent on co-expression with DAZL with FRET values being comparable between both cellular compartments. Thus, the functions of the germ plasm components may be influenced by interactions and sub-cellular localization.

## FRET Analysis of Intracellular Localization and Protein Interactions in Mouse and Human Embryonic Stem Cells

Recently it has been noted that many proteins that are expressed in early germ cells, are also expressed in mouse and human ESCs and have mutant phenotypes associated with ESC maintenance (for example, *Oct4* and *Nanog*) (Nichols et al., 1998; Chambers et al., 2003; Jaruzelska et al., 2003; Mitsui et al., 2003; Moore et al., 2003; Clark et al., 2004b; Moore et al., 2004). As development of the early mammalian embryo progresses, expression of these genes, along with homologs of germ plasm components, is then restricted to the nascent germ cells with little or no expression in somatic cells (Nichols et al., 1998; Lawson et al., 1999; Ying et al., 2001; Chambers et al., 2003; Jaruzelska et al., 2003; Mitsui et al., 2003; Moore et al., 2003; Tsuda et al., 2003; Clark et al., 2004b). Thus, the genes expressed in mouse and human ESCs and early germ cells form a largely overlapping set suggesting that these cell types may be closely-related (or identical) cell types or utilize common regulatory pathways (Moore et al., 2003; Clark et al., 2004a; Clark et al., 2004b; Moore et al., 2004; Zwaka and Thomson, 2005).

In light of the common gene expression in germ cells and ESCs, we next examined whether protein interactions between DAZL and PUM2 were also similar in these two cell types. We observed that the localization and FRET signals in transfected mESCs were in sharp contrast to those seen in germ cells (Fig. 4A). As shown in Fig. 4B and C (Panels I - VI), although we observed the co-expression of eGFP-DAZL with RFP-DAZL and eGFP-PUM2 with RFP-PUM2, we did not observe FRET indicative of homo-dimerization. Moreover, when eGFP-DAZL and RFP-PUM2 were expressed together, no interactions were detected via FRET (Fig. 4D; Panel I-VI). In addition, rather than being distributed throughout the cell in the presence of DAZL, PUM2 protein remained localized to cytoplasm (Fig. 4D; Panels IV-V) which was confirmed by staining the cell nuclei with Hoechst (Fig. S1). This observation supports the idea that movement of PUM2 into the nucleus may be dependent on dimerization with DAZL.

We extended our studies to examine whether DAZL and PUM2 proteins form homodimers and heterodimers in human embryonic stem cells, using the H9 line (NIH code: WA09). After co-transfection of DAZL fusion proteins, we observed that human embryonic stem cells readily expressed DAZL proteins throughout the cell, as has been observed with endogenous germ cell proteins (Clark et al., 2004a; Clark et al., 2004b); however, we observed no FRET between DAZL proteins indicating a lack of homodimer formation (Fig. 4E; Panels I - VI). Parallel results were observed with PUM2 fusion proteins. There was widespread expression of PUM2 in the cytoplasm but a complete absence of FRET indicative of PUM2 homo-dimerization (Fig. 4F; Panels I - VI). Finally, expression of DAZL and PUM2 fusions proteins together did not result in FRET and PUM2 movement into the nucleus of human embryonic stem cells (Fig. 4G; Panels I - VI).

## FRET Analysis of Intracellular Localization and Protein Interactions in Primordial Germ Cells

To this point, it was clear that we could distinguish germ cells of primary and immortalized cultures from human and mouse embryonic stem cells by distinct protein interactions, detectable by FRET. We next explored the status of DAZL and PUM2 protein interactions in embryonic germ cells to assess the timeline of complex formation. For this purpose, we used two low-passage embryonic germ cell lines generated from embryonic day E8.5 and E12.5 embryos. The embryonic germ cells were co-transfected with DAZL and PUM2 constructs as previously. In both E8.5 and E12.5 cells, we observed that the proteins localized throughout the germ cells (Fig. 4). However, we did not observe any FRET indicative of either DAZL or PUM2 homo-dimerization at either stage (Fig. 5B, C, E, F; Panels I - VI). The absence of signals was confirmed with acceptor/donor ratios of zero. However, in contrast to results of homo-dimerization, we observed that when both DAZL and PUM2 fusion proteins were

expressed together, the two proteins interacted, and the FRET/donor ratio increased with acceptor/donor ratio in co-transfected cells at both stages of development (Fig. 5D and G; Panels I - VI). The interaction was also associated with the detection of both DAZL and PUM2 proteins localized to the nucleus.

### FRET Analysis of Intracellular Localization and Protein Interactions in Somatic Cells

To determine whether the formation of PUM2 and DAZL homo- and/or heterodimers is a specific property of germ cells, we further examined the interactions of these proteins, in experiments that parallel those described above, in somatic cells ((human embryonic kidney (HEK) cells; data not shown) and mouse Leydig cells (Fig. 6A - C; Panels I - VI)). We observed no formation of homo- or hetero-dimers in somatic cells; moreover, PUM2 protein was not redistributed to the nucleus.

### Interacting Proteins Can Bind the Same 3' UTR of *SDAD1*

We noted that DAZL and PUM2 protein complexes are diagnostic of different stages of germ cell development, as described above, with postnatal germ cells possessing complexed homodimers and heterodimers of these factors. In addition, previous results with coimmunoprecipitation and the yeast two hybrid system, indicate direct interactions *in vitro* (Tsui et al., 2000a; Moore et al., 2003; Urano et al., 2005); (Xu et al., 2001; Jaruzelska et al., 2003; Moore et al., 2004). Taken together, with other published reports, this data suggests the presence of a complex or particle that contains RNA-binding proteins to regulate gene expression post-transcriptionally in germ cells beginning early in their development. This complex of proteins might potentially interact on the same RNA molecule and/or may simultaneously bind different RNA molecules. We used a yeast three hybrid system to test whether different RNA-binding proteins in the complexes could potentially recognize the same RNA substrate.

Based on reported interactions of multiple protein partners (Fig. 7A) (Tsui et al., 2000a; Xu et al., 2001; Moore et al., 2003; Jaruzelska et al., 2003; Moore et al., 2004; Urano et al., 2005; Collier et al., 2005; Padmanabhan and Richter, 2006), we hypothesized that different RNA-binding proteins could bind the 3'UTR of the *SDAD1* gene, one of several mRNAs that was previously identified as a target of DAZL and PUM2 proteins (Fox et al., 2005). When, we tested the ability of multiple interacting RNA binding proteins to bind 90 base pair overlapping fragments of the *SDAD1* 3'UTR sequences in a yeast system, we observed that PUM2, DAZL, BOULE, DAZ2 and DAZAP1 could potentially bind distinct sequences in the same RNA (Fig. 7B). We previously defined the PUM2 cis-elements via 5' and 3' deletion analysis of constructs derived from the 90 base pair fragments; subsequently, we confirmed the interactions by gel shift assays (Fox et al., 2005). In this way we mapped specific sequence requirements for PUM2 binding (Fig. 7C). Furthermore, we mapped two DAZL binding sequences by gel shift assay which together with other binding data provided a consensus sequence for DAZL (Fig. 7C). Thus, these data provide evidence that multiple RNA-binding proteins can recognize the same RNAs, with specificity demonstrated for two interacting partners, as also suggested by studies from other research groups on interactions of these factors and others, including the polyadenylate binding proteins in germ cells (Collier et al., 2005; Urano et al., 2005; Fox et al., 2005; Padmanabhan and Richter, 2006).

## Discussion

### Formation and Maintenance of Germ Cells in Mammals

In mammalian species, male and female germ cells are formed independently of germ plasm (Saffman and Lasko, 1999). Fate mapping studies of the pre-implantation mouse epiblast have revealed that mouse germ cells are specified in the proximal epiblast in response to signals



from the neighboring extraembryonic ectoderm, in particular BMP4 signaling (Tam and Zhou, 1996; Fujiwara et al., 2001). Yet, the proximal epiblast is not predestined to a germ cell fate; transplantation of distal epiblast to contact extraembryonic ectoderm also results in germ cell formation (Tam and Zhou, 1996). Thus, it is the extraembryonic ectoderm that provides one of the first signals for germ cell specification in the epiblast, not an autonomous signal from within the epiblast. Germ cells are then first recognized at 7.2 days post coitum (dpc) as an extraembryonic cluster of cells that express tissue non-specific alkaline phosphatase, *Oct4*, and *Stella*, at the base of the allantois following gastrulation (Chiquoine, 1954; Scholer et al., 1990a; Scholer et al., 1990b; Saitou et al., 2002). Genes that are required for the formation of primordial germ cells such as *Blimp1*, a transcriptional repressor, may act by repressing expression of somatic genes (Ohinata et al., 2005).

The embryological period equivalent to mouse E5.5-E7.2 in human embryo development occurs shortly after implantation. Thus, the analysis of human germ cell specification *in vivo* is impracticable. However, recent studies have indicated that mouse and human ESCs (derived from the inner cell mass of the blastocyst prior to epiblast formation) are capable of differentiating into female and male germ cells *in vitro* (Hubner et al., 2003; Toyooka et al., 2003; Clark et al., 2004a; Clark et al., 2004b; Geijsen et al., 2004). A common theme noted in studies on differentiation from ESCs has been the observation that it is very difficult to distinguish ESCs from early germ cell populations, prior to erasure and sex-specific establishment of imprinting, by the usual techniques such as morphology, mRNA and protein expression. Indeed, genes once thought to be germ cell specific are widely expressed in all cells in mouse and human ESC cultures (Moore et al., 2003; Abeyta et al., 2004; Clark et al., 2004a; Clark et al., 2004b; Moore et al., 2004). These observations, along with similarities in the biological properties of ESCs and primordial germ cells in terms of pluripotency and differentiation have prompted speculation on the origins of embryonic stem cells, including suggestions that embryonic stem cells may have a germ cell origin (Evans and Hunter, 2002; Buehr and Smith, 2003; Zwaka and Thomson, 2005).

### DAZL and the Human Germ Cell Lineage

Previously, we showed that undifferentiated human embryonic stem cells expressed *DAZL* and associated proteins but not later germ cell markers such as *VASA*. Given this expression pattern and the observation that in diverse organisms including flies, worms, frogs, fish, mice and humans, *DAZL* expression is restricted to germ cells, we proposed that *DAZL* is one of the earliest known marker of the germ cell lineage in humans and that human embryonic stem cells may have in part or total been derived from early germ cell precursors (Xu et al., 2001; Clark et al., 2004a; Clark et al., 2004b). Here we demonstrate that there are clear differences between germ cells and embryonic stem cells that can be observed by FRET. Although *DAZL* and *PUM2* proteins are expressed uniformly in human and mouse ESCs, we observed no direct interaction of *DAZL* and *PUM2* in homodimers or heterodimers in this cell type. In contrast, PGCs are characterized by association of *DAZL* and *PUM2* in heterodimers and premeiotic, postnatal germ cells were observed to have FRET indicative of both *DAZL* and *PUM2* homodimers and heterodimers.

In many species, elegant studies have demonstrated that the critical step to establishing and maintaining germ cells is the assembly of protein complexes that make up the germ plasm (Santos and Lehmann, 2004). *DAZL* and *PUM2* proteins are known to be components of germ plasm in diverse species (Santos and Lehmann, 2004). Thus, we suggest that although obviously less distinct by microscopy and more protracted in assembly, our data indicates that protein complexes are assembled, from homologs of germ plasm components, in living mammalian germ cells. Based on these observations and several novel findings that are emerging from studies of ESC-derived germ cells, we propose a model of germ cell formation.



Several studies have indicated that mammalian ESCs, including hESCs and inner cell mass cells are not equivalent and that hESCs may be closely related to primordial germ cells (Evans and Hunter, 2002; Clark et al., 2004a; Zwaka and Thomson, 2005). Thus, we propose that proteins required for initial formation and/or maintenance of nascent germ cells are translated in ESCs. Then, analogous to *in vivo* experiments, in response to extracellular signaling, protein components may interact to form complexes (or germ cell particles) that function in formation of the germ cell lineage and/or maintenance of early populations of germ cells. Ultimately, the formation of such complexes, may commit the cells to the germ cell lineage, at least under physiological conditions so that differentiate to mature gametes is the normal pathway (Fig. 8).

This model remains to be tested *in vivo* and additional data should be gathered via *in vitro* differentiation, as well. The model suggests that factors that are present in ESCs and/or germ cells may regulate complex assembly, perhaps through protein modifications, such as phosphorylation. In addition, we suggest that co-fractionating proteins may be present in one or more complexes and that additional components remain to be identified, including the RNAs that are present. Most importantly, the function of the complex in regulating germ cell specific RNA metabolism remains to be determined, though recent studies are beginning to shed light on this (Venables et al., 2001; Jiao et al., 2002; Collier et al., 2005; Fox et al., 2005; Reynolds et al., 2005; Padmanabhan and Richter, 2006). Nevertheless these studies demonstrate that PUM2 and DAZL are constituents of a ribonucleoprotein complex within living germ cells and hypothesize that the complex plays a fundamental role in early germ cell formation and/or maintenance. Finally, we note the similarities in constitution of the complexes described and that of the germ plasm of model organisms such as *Xenopus*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We thank Andrew Feinberg and Patricia Labosky for the mouse EG lines from E8.5 E12.5 embryos, respectively. We thank Roger Tsien for the monomeric red fluorescent protein, Marvin Wickens for yeast three hybrid components, Martin Dym for the spermatogonial stem cell resources, Pauline Yen for DAZ-2 cDNA and DAZAP-1 antibody, Nigel Killen for the mouse E14 embryonic stem cell line, Sindy Mellon for the mouse Leydig cell line, and Anna Bogdanova for assistance with mice. We also thank Haifan Lin and Neils Geijsens for helpful comments on the manuscript, as well as members of the Reijo Pera laboratory. This work was supported by the Lalor Foundation (M.F.), a National Institute of Health National Research Award fellowship (to J.U.) and by grants from the National Institutes of Health (RO1 HD037095, RO1 HD047221 and R24 RR017498-03), the Sandler Family Foundation, the American Stem Cell Research Foundation and the California TRDRP (to R.A.R.P.).

## References

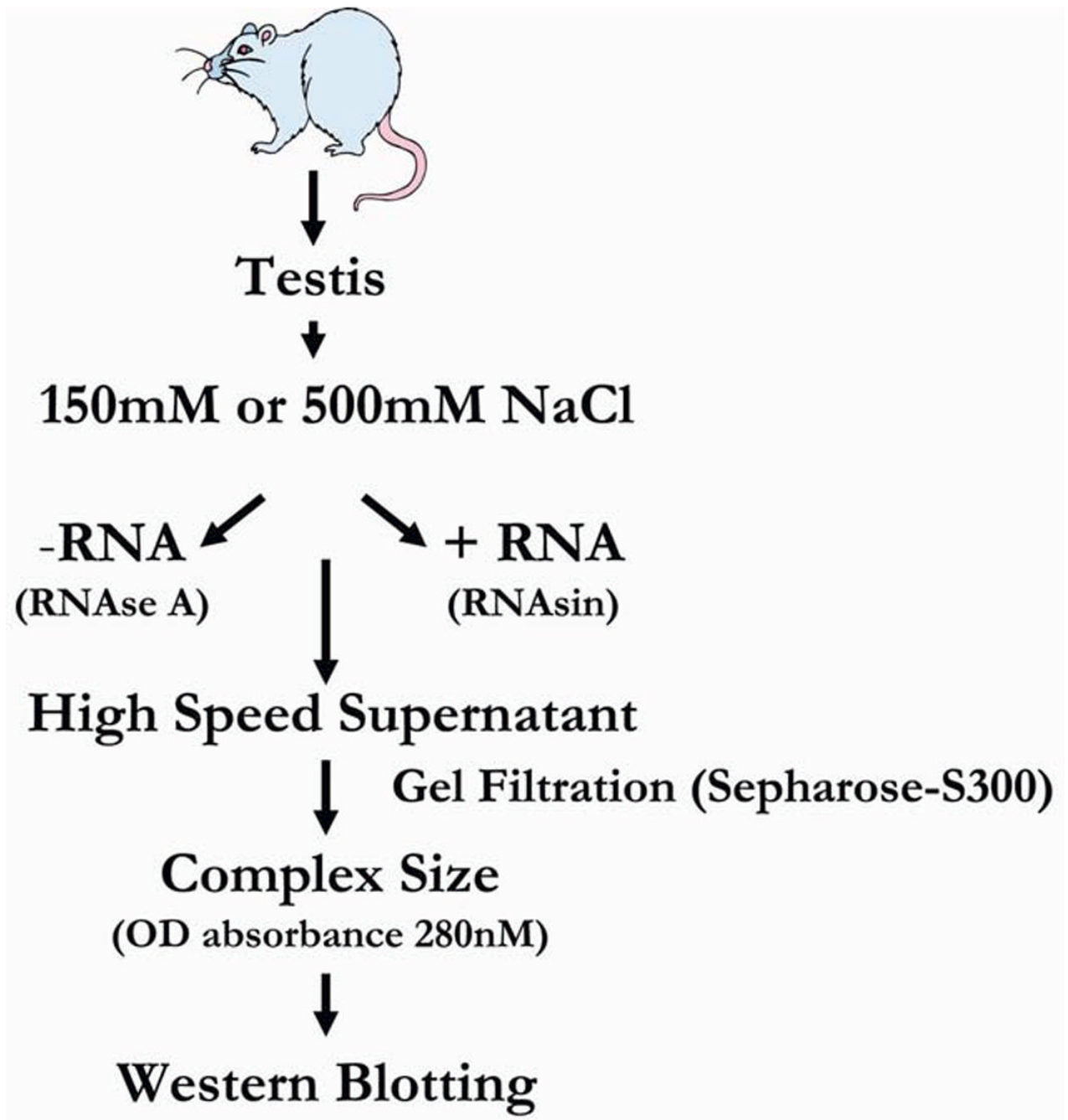
- Abeyta M, Clark AT, Rodriguez R, Bodnar M, Reijo Pera RA, Firpo M. Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum Mol Genet* 2004;13:601–608. [PubMed: 14749348]
- Asaoka-Taguchi M, Yamada M, Nakamura A, Hanyu K, Kobayashi S. Maternal Pumilio acts together with Nanos in germline development in *Drosophila* embryos. *Nature Cell Biol* 1999;1:431–7. [PubMed: 10559987]
- Buehr M, Smith A. Genesis of embryonic stem cells. *Philos Trans R Soc Lond B Biol Sci* 2003;358:1397–1402. [PubMed: 14511487]
- Campbell R, Tour O, Palmer A, Steinbach P, Baird G, Zacharias D, Tsien R. A monomeric red fluorescent protein. *Proc Natl Acad Sci* 2002;99:7877–7882. [PubMed: 12060735]
- Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. *Proc Natl Acad Sci* 2000;97:9585–9590. [PubMed: 10920202]

- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of *Nanog*, a pluripotency sustaining factor in embryonic stem cells. *Cell* 2003;113:643–655. [PubMed: 12787505]
- Chiquoine A. The identification, origin and migration of the primordial germ cells in the mouse embryo. *Anat Rec* 1954;118:135–146. [PubMed: 13138919]
- Clark AT, Bodnar MS, Fox MS, Rodriguez RT, Abeyta MJ, Firpo MT, Reijo Pera RA. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet* 2004a;13:727–739. [PubMed: 14962983]
- Clark AT, Rodriguez R, Bodnar M, Abeyta M, Turek P, Firpo M, Reijo Pera RA. Human *STELLAR*, *NANOG*, and *GDF3* genes are expressed in pluripotent cells and map to chromosome 12p13, a hot-spot for teratocarcinoma. *Stem Cells* 2004b;22:169–179. [PubMed: 14990856]
- Collier B, Gorgoni B, Loveridge C, Cooke H, Gray N. The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. *EMBO J* 2005;24:2656–2666. [PubMed: 16001084]
- D'Agostino I, Merritt C, Chen P, Seydoux G, Subramaniam K. Translational repression restricts expression of the *C. elegans* *Nanos* homolog *NOS-2* to the embryonic germline. *Dev Biol* 2006;292:244–252. [PubMed: 16499902]
- Day R, Schaufele F. Imaging molecular interactions in living cells. *Mol Endocrinol* 2005;19:1675–1686. [PubMed: 15761028]
- Eberhart CG, Maines JZ, Wasserman SA. Meiotic cell cycle requirement for a fly homologue of human *Deleted in AZOOSPERMIA*. *Nature* 1996;381:783–785. [PubMed: 8657280]
- Evans M, Hunter S. Source and nature of embryonic stem cells. *C R Biol* 2002;325:1003–7. [PubMed: 12494496]
- Feng LX, Chen Y, Dettin L, Reijo Pera RA, Herr JC, Goldberg E, Dym M. Generation and in vitro differentiation of a spermatogonial cell line. *Science* 2002;297:392–395. [PubMed: 12077424]
- Forbes A, Lehmann R. Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development* 1998;125:679–690. [PubMed: 9435288]
- Fox M, Urano J, Reijo Pera RA. Identification and characterization of RNA sequences to which human PUMILIO-2 (PUM2) and Deleted in Azoospermia-Like (DAZL) bind. *Genomics* 2005;85:92–105. [PubMed: 15607425]
- Fujiwara T, Dunn NR, Hogan BL. Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. *Proc Natl Acad Sci* 2001;98:13739–13744. [PubMed: 11707591]
- Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 2004;427:148–154. [PubMed: 14668819]
- Houston DW, King ML. A critical role for *Xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. *Development* 2000;127:447–456. [PubMed: 10631166]
- Howley C, Ho RK. mRNA localization patterns in zebrafish oocytes. *Mech Dev* 2000;92:305–309. [PubMed: 10727871]
- Hubner K, Fuhrmann G, Christenson L, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss J III, Boiani M, Scholer H. Derivation of oocytes from mouse embryonic stem cells. *Science* 2003;300:1251–1256. [PubMed: 12730498]
- Ikenishi K. Germ plasm in *Caenorhabditis elegans*, *Drosophila* and *Xenopus*. *Dev Growth Differ* 1998;40:1–10. [PubMed: 9563905]
- Jaruzelska J, Kotecki M, Kusz K, Spik A, Firpo M, Reijo RA. Conservation of a Pumilio-Nanos complex from *Drosophila* germ plasm to human germ cells. *Dev Genes Evol* 2003;213:120–126. [PubMed: 12690449]
- Jiao X, Trifillis P, Kiledjian M. Identification of target messenger RNA substrates for the murine Deleted in Azoospermia-Like RNA-Binding Protein. *Biol Reprod* 2002;66:475–485. [PubMed: 11804965]
- Johnson AD, BR, Drum M, Masi T. Expression of *axolotl dazl* rna, a marker of germ plasm: widespread maternal rna and onset of expression in germ cells approaching the gonad. *Dev Biol* 2001;234:402–415. [PubMed: 11397009]
- Karashima T, Sugimoto A, Yamamoto M. *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not for spermatogenesis. *Development* 2000;127:1069–1079. [PubMed: 10662646]

- Koprunner M, Thisse C, Thisse B, Raz E. A zebrafish nanos-related gene is essential for the development of primordial germ cells. *Genes Dev* 2001;15:2877–2885. [PubMed: 11691838]
- Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol Reprod* 2004;71:722–731. [PubMed: 15115718]
- Labosky P, Barlow D, Hogan B. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of *insulin-like growth factor 2 receptor (Igf2r)* gene compared with embryonic stem (ES) cell lines. *Development* 1994;120:3197–3204. [PubMed: 7720562]
- Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL. *Bmp4* is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* 1999;13:424–436. [PubMed: 10049358]
- Lin H, Spradling AC. A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* 1997;124:2463–76. [PubMed: 9199372]
- Lin Y, Page D. Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. *Dev Biol* 2005;288:309–316. [PubMed: 16310179]
- Maegawa S, Yasuda K, Inoue K. Maternal mRNA localization of zebrafish *DAZ-like* gene. *Mech. Dev* 1999;81:223–226. [PubMed: 10330505]
- McLean DJ, Friel PJ, Johnston DS, Griswold MD. Characterization of spermatogonial stem cell maturation and differentiation in neonatal mice. *Biol Reprod* 2003;69:2085–2091. [PubMed: 12954735]
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 2003;113:631–642. [PubMed: 12787504]
- Mochizuki K, Nishimiya-Fujisawa C, Fujisawa T. Universal occurrence of the vasa-related genes among metazoans and their germline expression in *Hydra*. *Dev Genes Evol* 2001;211:299–308. [PubMed: 11466525]
- Moore FL, Jaruzelska J, Dorfmann D, Reijo Pera RA. Identification of a novel gene, *DZIP (DAZ Interacting Protein)*, that encodes a protein that interacts with DAZ and DAZL (Deleted in AZoospermia and DAZ-Like) and is expressed in embryonic stem cells and germ cells. *Genomics* 2004;83:834–843. [PubMed: 15081113]
- Moore FL, Jaruzelska J, Fox MS, Urano J, Firpo MT, Turek PJ, Dorfman DM, Reijo Pera RA. Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with DAZ (Deleted in AZoospermia) and DAZ-Like proteins. *Proc Natl Acad Sci* 2003;100:538–543. [PubMed: 12511597]
- Nakahata S, Katsu Y, Mita K, Inoue K, Nagahama Y, Yamashita M. Biochemical identification of *Xenopus* Pumilio as a sequence-specific cyclin B1 mRNA-binding protein that physically interacts with a Nanos homolog, Xcat-2, and a cytoplasmic polyadenylation element-binding protein. *J. Biol. Chem* 2001;276:20945–20953. [PubMed: 11283000]
- Nayernia K, Nolte J, Michelmann H, Lee J, Rathack K, Drusenheimer N, Dev A, Wulf G, Ehrmann I, Elliott D, Okpanyi V, Zechner U, Haaf T, Meinhardt A, Engel W. *In vitro*-differentiated embryonic stem cell give rise to male gametes that can generate offspring mice. *Dev Cell* 2006;11:125–132. [PubMed: 16824959]
- Nichols J, Zevnick B, Anastasiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct-4. *Cell* 1998;95:379–391. [PubMed: 9814708]
- Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton S, Obukhanych T, Nussenzweig M, Tarakhovsky A, Saitou M, Surani M. *Blimp1* is a critical determinant of the germ cell lineage in mice. *Nature* 2005;436:207–13. [PubMed: 15937476]
- Onyango P, Jiang S, Uejima H, Shamblott MJ, Gearhart JD, Cui H, Feinberg AP. Monoallelic expression and methylation of imprinted genes in human and mouse embryonic germ cell lineages. *Proc Natl Acad Sci* 2002;99:10599–10604. [PubMed: 12114541]
- Padmanabhan K, Richter J. Regulated Pumilio-2 binding controls RINGO/Spy mRNA translation and CPEB activation. *Genes Dev* 2006;20:199–209. [PubMed: 16418484]

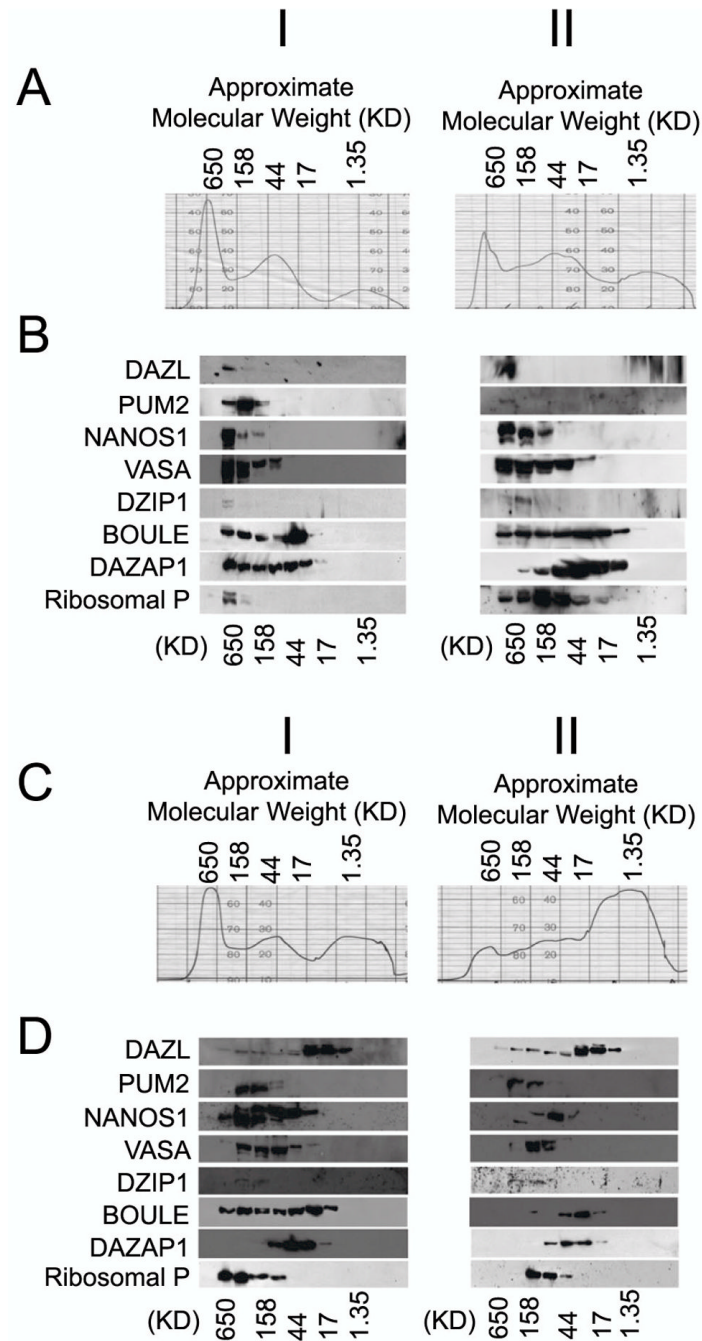
- Parisi M, Lin H. The *Drosophila Pumilio* gene encodes two functional protein isoforms that play multiple roles in germline development, gonadogenesis, oogenesis, and embryogenesis. *Genetics* 1999;153:235–250. [PubMed: 10471709]
- Reijo R, Alagappan RK, Patrizio P, Page DC. Severe oligospermia resulting from deletions of the *Azoospermia Factor* gene on the Y chromosome. *Lancet* 1996;347:1290–1293. [PubMed: 8622504]
- Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, Chapelle A.d.I. Silber S, Page DC. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet* 1995;10:383–393. [PubMed: 7670487]
- Reynolds N, Collier B, Maratou K, Bingham V, Speed R, Taggart M, Semple C, NK NG, Cooke H. Dazl binds in vivo to specific transcripts and can regulate the pre-meiotic translation of Mvh in germ cells. *Hum Mol Genet* 2005;14:3899–3909. [PubMed: 16278232]
- Ruggiu M, Cooke H. *In vivo* and *in vitro* analysis of homodimerisation activity of the mouse Dazl protein. *Gene* 2000;252:119–126. [PubMed: 10903443]
- Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke H. The mouse *Dazla* gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* 1997;389:73–77. [PubMed: 9288969]
- Saffman EE, Lasko P. Germline development in vertebrates and invertebrates. *Cell. Mol. Life. Sci* 1999;55:1141–1163. [PubMed: 10442094]
- Saitou M, Barton SC, Surani MA. A molecular programme for the specification of germ cell fate in mice. *Nature* 2002;418:293–300. [PubMed: 12124616]
- Santos A, Lehmann R. Germ cell specification and migration in *Drosophila* and beyond. *Curr Biol* 2004;14:R578–R589. [PubMed: 15268881]
- Scholer H, Dressler G, Balling R, Rohdewohld H, Gruss P. Oct-4: a germ line specific transcription factor mapping to the mouse t-complex. *EMBO J* 1990a;9:2185–2195. [PubMed: 2357966]
- Scholer H, Ruppert S, Suzuki N, Chowdhury K, Gruss P. New type of POU domain in germ line-specific protein Oct-4. *Nature* 1990b;344:435–439. [PubMed: 1690859]
- Subramaniam K, Seydoux G. *nos-1* and *nos-2*, two genes related to *Drosophila nanos*, regulate primordial germ cell development and survival in *Caenorhabditis elegans*. *Development* 1999;126:4861–4871. [PubMed: 10518502]
- Tam P, Zhou S. The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by position of the cells in the gastrulating mouse embryo. *Dev Biol* 1996;178:124–132. [PubMed: 8812114]
- Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T. The mouse homolog of *Drosophila Vasa* is required for the development of male germ cells. *Genes Dev* 2000;14:841–853. [PubMed: 10766740]
- Toyooka Y, Tsunekawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells *in vitro*. *Proc Natl Acad Sci* 2003;100:11457–11462. [PubMed: 14504407]
- Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S, Saga Y. Conserved role of nanos proteins in germ cell development. *Science* 2003;301:1239–1241. [PubMed: 12947200]
- Tsui S, Dai T, Roettger S, Schempp W, Salido EC, Yen PH. Identification of two novel proteins that interact with germ-cell specific RNA-binding proteins DAZ and DAZL1. *Genomics* 2000a;65:266–273. [PubMed: 10857750]
- Tsui S, Dai T, Warren ST, Salido EC, Yen PH. Association of the mouse infertility factor *DAZL1* with actively translating polyribosomes. *Biol. Reprod* 2000b;62:1655–1660. [PubMed: 10819768]
- Tung JY, Rosen MP, Nelson LM, Turek PJ, Witte JS, Cramer DW, Cedars MI, Reijo Pera RA. Variants in Deleted in AZoospermia-Like (DAZL) are correlated with reproductive parameters in men and women. *Hum Genet* 2006;118:730–740. [PubMed: 16328470]
- Urano J, Fox M, Reijo Pera RA. Interaction of the conserved meiotic regulators, BOULE (BOL) and PUMILIO-2 (PUM2). *Mol Reprod Dev* 2005;71:290–298. [PubMed: 15806553]
- Venables JP, Ruggiu M, Cooke HJ. The RNA binding specificity of the mouse Dazl protein. *Nucl Acids Res* 2001;29:2479–2483. [PubMed: 11410654]

- Weatherman R, Chang C, Clegg N, Carroll D, Day R, Baxter J, McDonnell D, Scanlan T, Schaufele F. Ligand-selective interactions of ER detected in living cells by fluorescence resonance energy transfer. *Mol Endocrinol* 2002;16:487–496. [PubMed: 11875107]
- Wylie C. Germ cells. *Curr Opin Genet Dev* 2000;10:410–3. [PubMed: 10889067]
- Xu EY, Moore FL, Reijo Pera RA. A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in all metazoans. *Proc Natl Acad Sci* 2001;98:7414–7419. [PubMed: 11390979]
- Ying Y, Qi X, Zhao G-Q. Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. *Proc Natl Acad Sci* 2001;98:7858–62. [PubMed: 11427739]
- Zhou Y, King M. Sending RNAs into the future: RNA localization and germ cell fate. *IUBMB Life* 2004;56:19–27. [PubMed: 14992376]
- Zwaka T, Thomson J. A germ cell origin of embryonic stem cells? *Development* 2005;132:227–233. [PubMed: 15623802]

**Fig 1.**

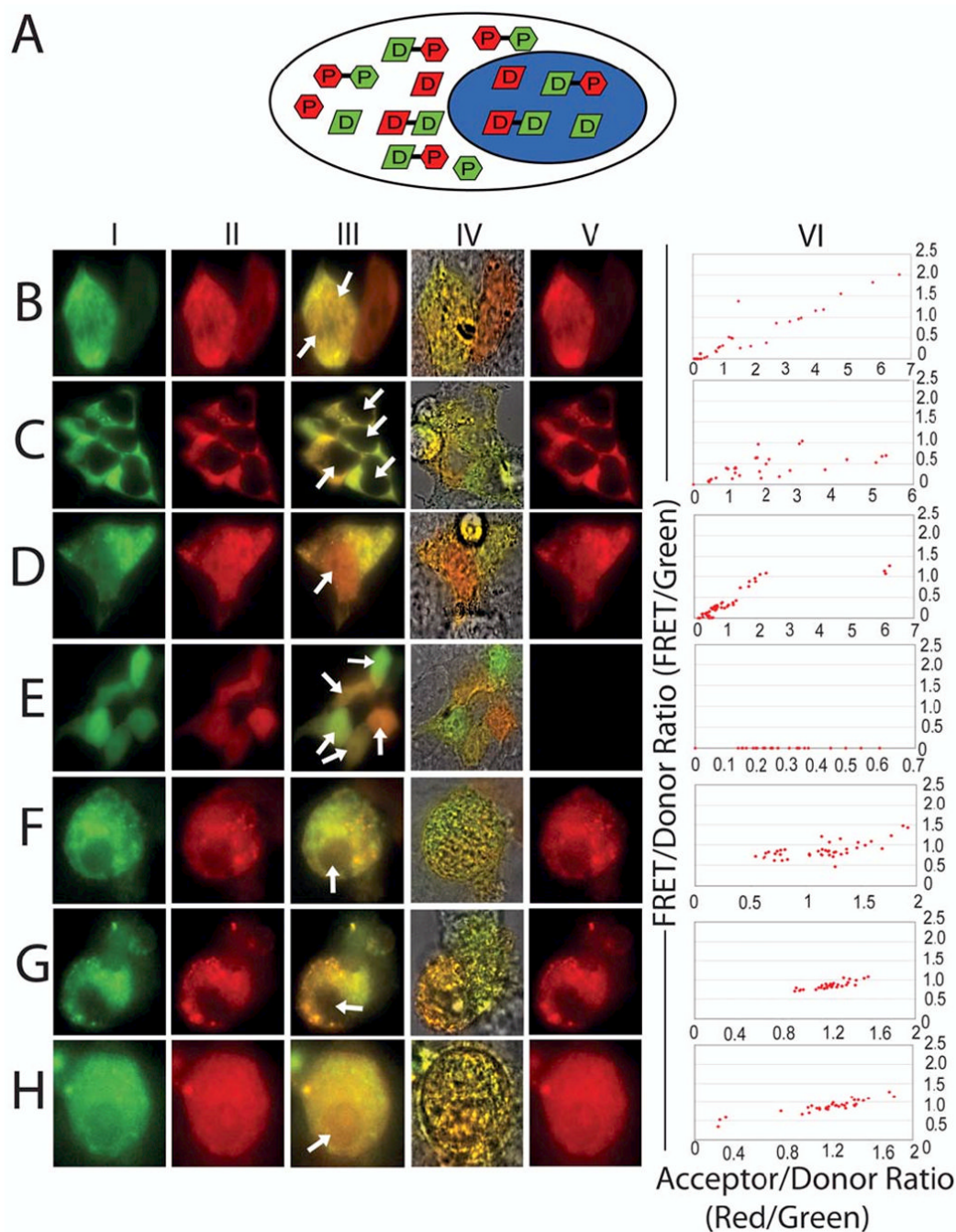
Schematic of methods used to analyze protein complexes in germ cells. As shown, mouse testis extract was prepared in the presence of either RNasin or RNase A in both physiological and high salt conditions (500 mM NaCl). To examine whether DAZL and PUM2 participate in the formation of one or several complexes *in vivo*, cytoplasmic extracts were then subjected to size exclusion chromatography and components of the eluants were analyzed by Western blotting. The relative contributions of protein-RNA versus protein-protein interactions can be approximated via comparison of protein complex profiles from different treatments.



**Fig. 2.**

Protein complexes revealed by size exclusion chromatography. (A) Cytoplasmic lysate from mouse testis treated with RNasin (I) or RNase A (II) in physiological salt concentrations (150mM NaCl) was fractionated by size exclusion chromatography; absorbance readings at 280nm for each 0.5 ml-fraction were plotted. (B) The presence of DAZL, PUM2, NANOS1, VASA, BOULE, DAZAP1, and Ribosomal P proteins was visualized by Western blotting of proteins in extracts treated with either RNasin (I) or RNase A (II) in physiological salt concentrations (150mM NaCl). The position of molecular-weight makers separated in parallel on the same column is indicated below the Western blot. (C) Cytoplasmic lysate from mouse testis treated with RNasin (I) or RNase A (II) was fractionated by size exclusion

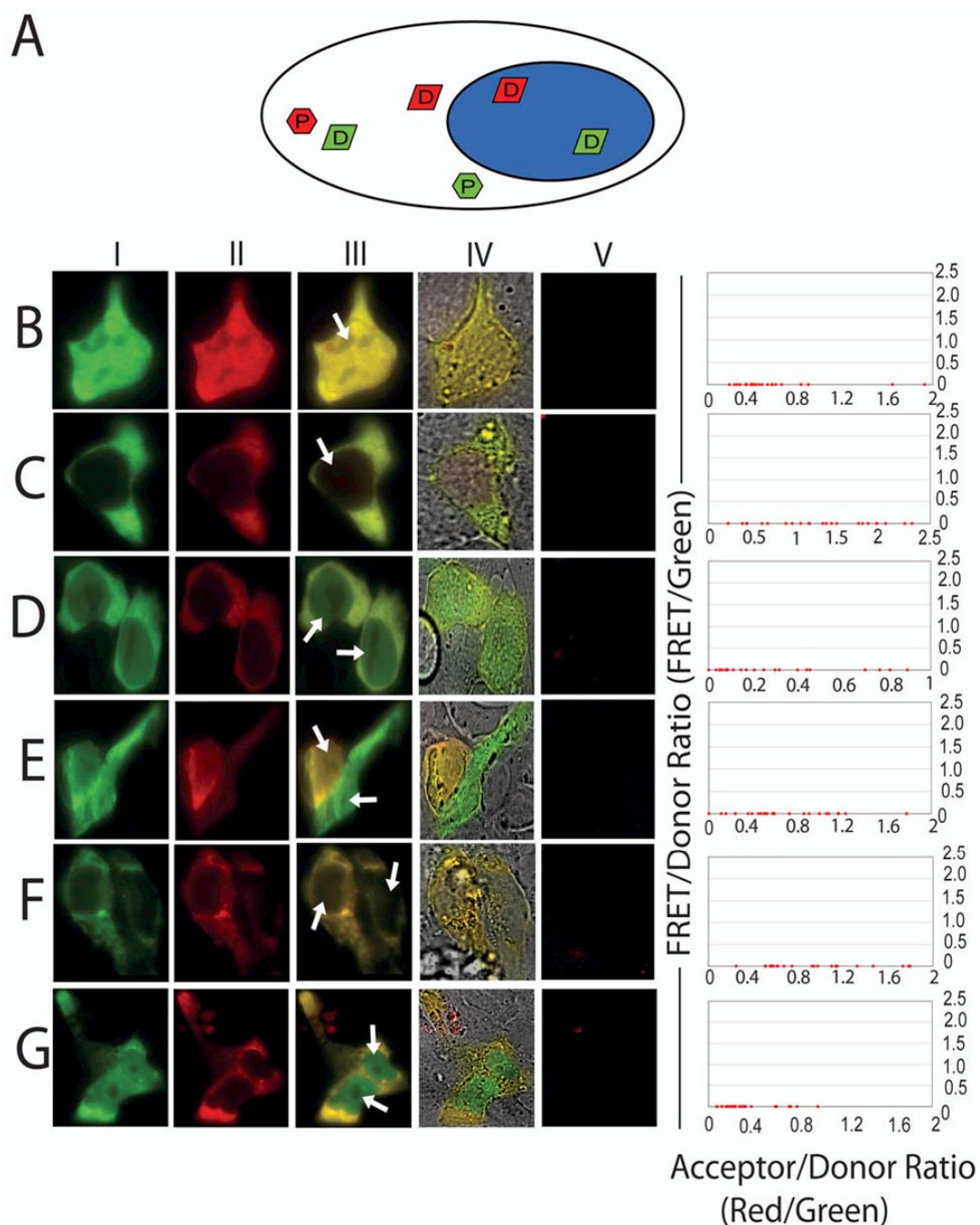
chromatography in high salt concentrations (500mM NaCl); absorbance readings at 280nm for each fraction were plotted. (D) The presence of DAZL, PUM2, NANOS1, VASA, BOULE, DAZAP1, and Ribosomal P proteins was visualized by Western blotting of proteins in extracts treated with either RNasin (I) or RNase A (II) in high salt concentrations (500mM NaCl). The position of molecular-weight markers separated in parallel on the same column is indicated below the Western blot. Molecular weight markers were thyroglobulin (670 KD), gamma-globulin (158 KD), ovalbumin (44 KD), and Vitamin B<sub>12</sub> (1.3 KD).



**Fig. 3.**

DAZL and PUM2 proteins in living postnatal mouse germ cells. (A) Reference diagram of a postnatal germ cell with the nucleus colored in blue. Cellular distribution of DAZL and PUM2 subunits are depicted by the letters D and P respectively; D-D and P-P correspond to homodimers while D-P is used to depict DAZL/PUM2 complexes. (B) eGFP-DAZL interaction with RFP-DAZL in spermatogonial stem cells. (C) eGFP-PUM2 interaction with RFP-PUM2 in spermatogonial stem cells. (D) eGFP-DAZL interaction with RFP-PUM2 in spermatogonial stem cells. (E) Negative control eGFP interaction with RFP in spermatogonial stem cells. (F) eGFP-DAZL interaction with RFP-DAZL in primary germ cells from day 3 mice. (G) eGFP-PUM2 interaction with RFP-PUM2 in primary germ cells from day 3 mice.

(H) eGFP-DAZL interaction with RFP-PUM2 in primary germ cells from day 3 mice. Panels I - VI in each row as follows: (I) A reference image showing expression of eGFP fusion constructs throughout the stem cell. (II) A second digital image obtained from the same plane showing expression of RFP fusion construct. (III) Colocalization of panels I and II depicted in yellow. (IV) Phase contrast overlay of Panels I and II. (V) FRET detected by stimulating the cells with blue light and using the acceptor filter for the RFP protein. (VI) FRET/donor ratios as graphed as a function of acceptor/donor ratios.

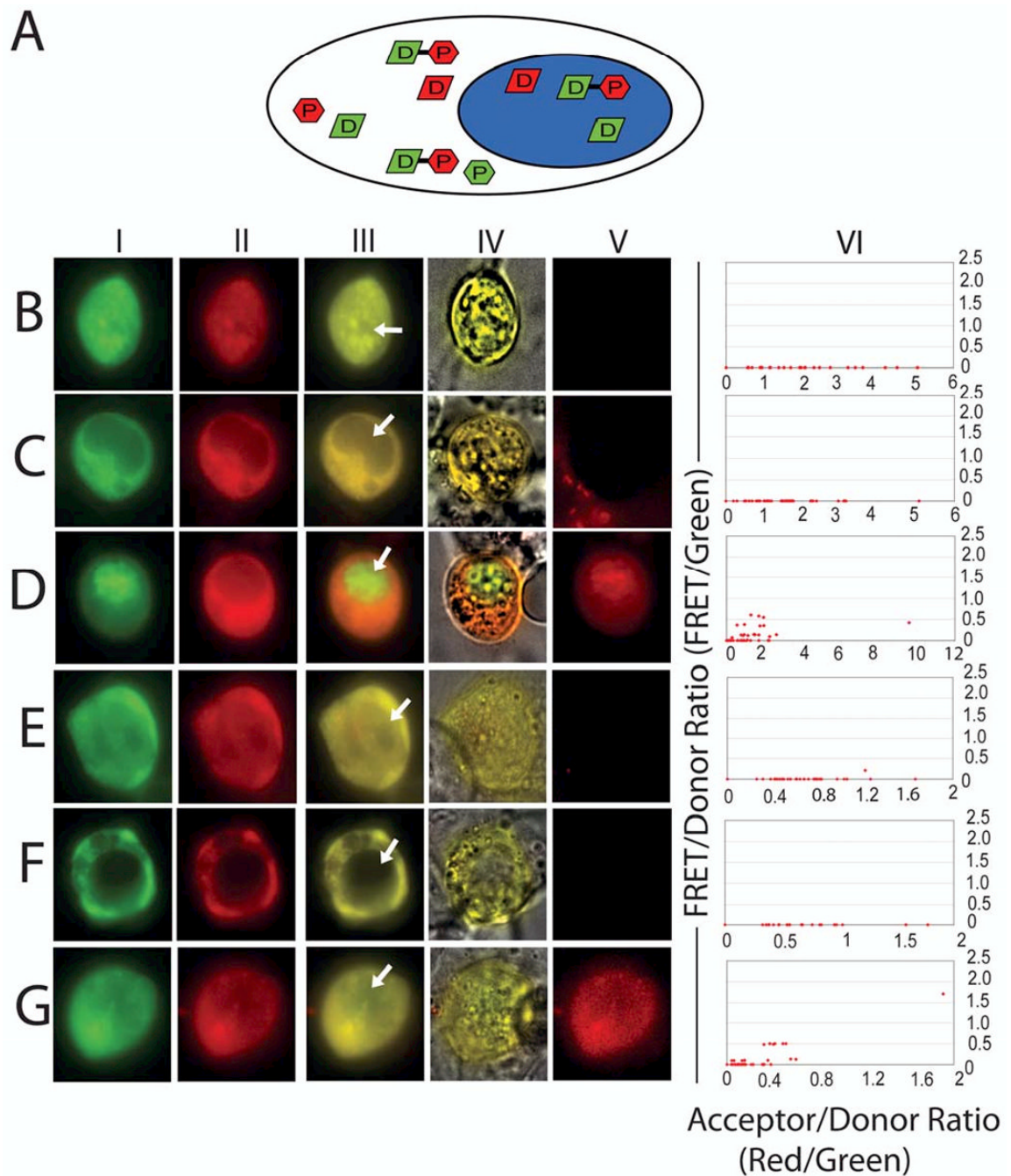


**Fig. 4.**

DAZL and PUM2 proteins in living embryonic stem cells. (A) Reference diagram of a embryonic stem cell with the nucleus colored in blue. Cellular distribution of DAZL and PUM2 subunits are depicted by the letters D and P respectively. (B) eGFP-DAZL interaction with RFP-DAZL in mouse embryonic stem cells. (C) eGFP-PUM2 interaction with RFP-PUM2 in mouse embryonic stem cells. (D) eGFP-DAZL interaction with RFP-PUM2 in mouse embryonic stem cells. (E) eGFP-DAZL interaction with RFP-DAZL in human embryonic stem cells. (F) eGFP-PUM2 interaction with RFP-PUM2 in human embryonic stem cells. (G) eGFP-DAZL interaction with RFP-PUM2 in human embryonic stem cells. Panels I - VI in each row as follows: (I) A reference image showing expression of eGFP fusion constructs throughout

the stem cell. (II) A second digital image obtained from the same plane showing expression of RFP fusion construct. (III) Colocalization of panels I and II depicted in yellow. (IV) Phase contrast overlay of Panels I and II. (V) FRET detected by stimulating the cells with blue light and using the acceptor filter for the RFP protein. (VI) FRET/donor ratios as graphed as a function of acceptor/donor ratios.

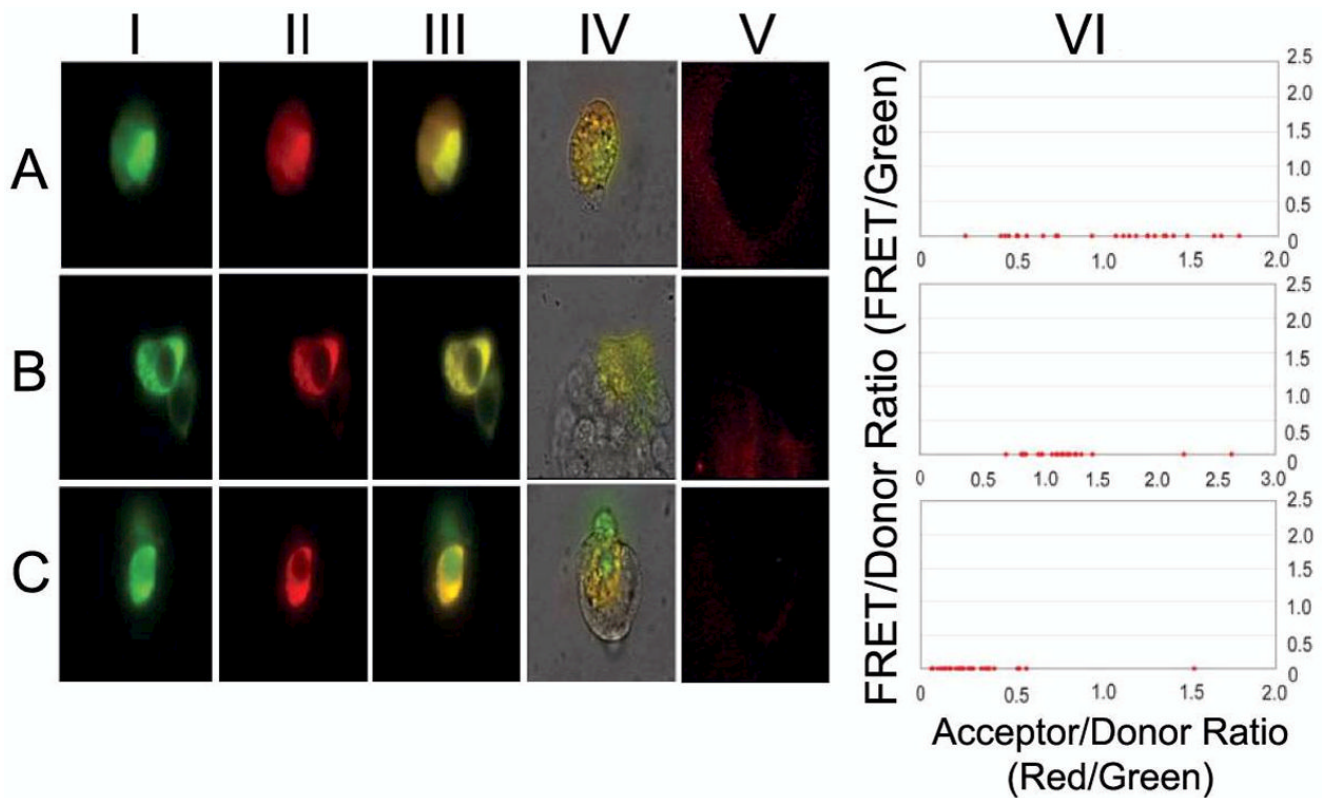




**Fig. 5.** DAZL and PUM2 proteins in living mouse embryonic germ cells from day 8.5 and 12.5 embryos. (A) Reference diagram of a postnatal germ cell with the nucleus colored in blue. Cellular distribution of DAZL and PUM2 subunits are depicted by the letters D and P respectively while D-P is used to depict DAZL/PUM2 complexes. (BA) eGFP-DAZL interaction with RFP-DAZL in embryonic germ cells from day 8.5. (C) eGFP-PUM2 interaction with RFP-PUM2 in embryonic germ cells from day 8.5. (D) eGFP-DAZL interaction with RFP-PUM2 in embryonic germ cells from day 8.5. (E) eGFP-DAZL interaction with RFP-DAZL in embryonic germ cells from day 12.5. (F) eGFP-PUM2 interaction with RFP-PUM2 in embryonic germ cells from day 12.5. (G) eGFP-DAZL

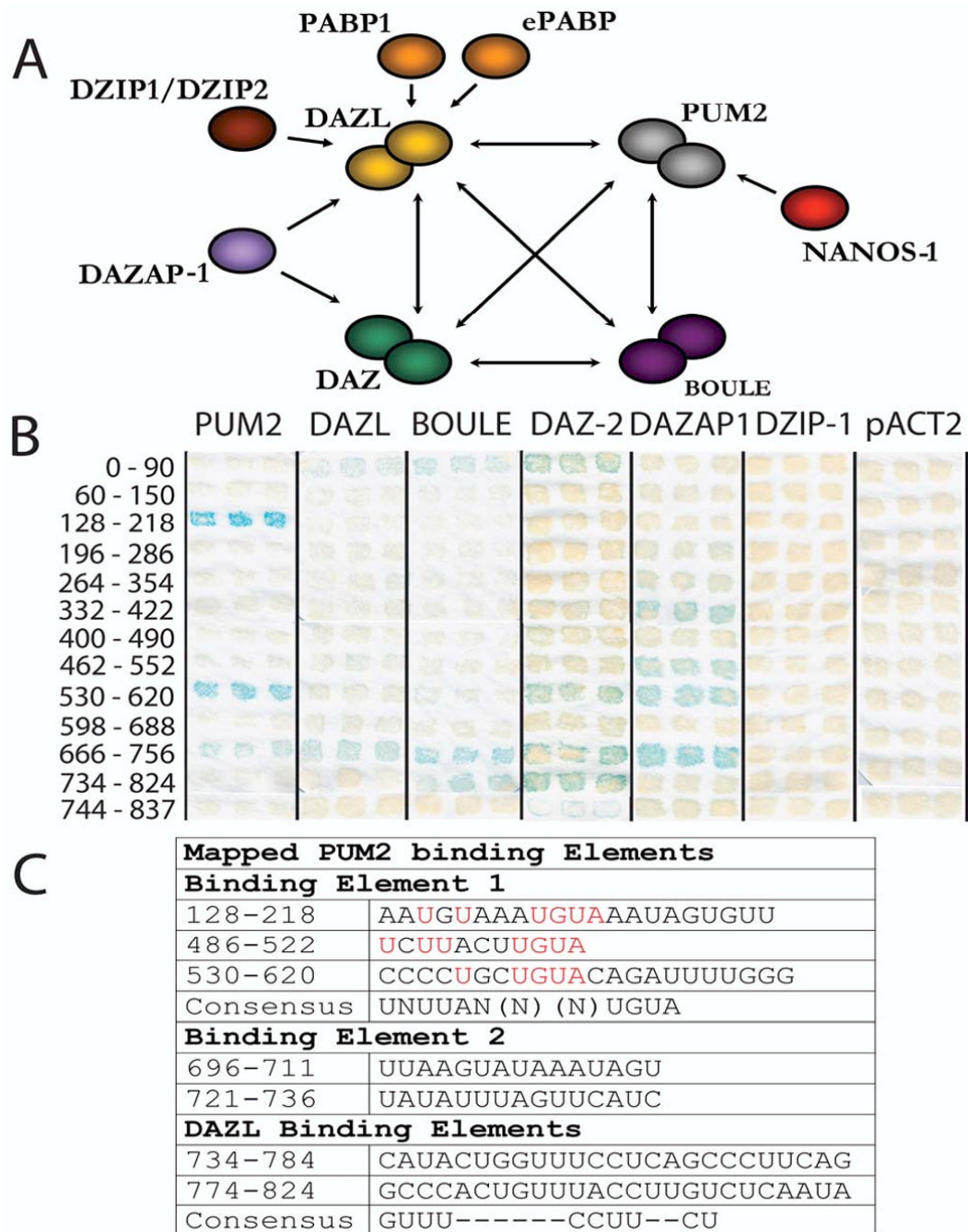
interaction with RFP-PUM2 in embryonic germ cells from day 12.5. Panels I - VI in each row as follows: (I) A reference image showing expression of eGFP fusion constructs throughout the stem cell. (II) A second digital image obtained from the same plane showing expression of RFP fusion construct. (III) Colocalization of panels I and II depicted in yellow. (IV) Phase contrast overlay of Panels I and II. (V) FRET detected by stimulating the cells with blue light and using the acceptor filter for the RFP protein. (VI) FRET/donor ratios as graphed as a function of acceptor/donor ratios.

(B) eGFP-DAZL interaction with RFP-DAZL in embryonic germ cells from day 8.5. (C) eGFP-PUM2 interaction with RFP-PUM2 in embryonic germ cells from day 8.5. (D) eGFP-DAZL interaction with RFP-PUM2 in embryonic germ cells from day 8.5. (E) eGFP-DAZL interaction with RFP-DAZL in embryonic germ cells from day 12.5. (F) eGFP-PUM2 interaction with RFP-PUM2 in embryonic germ cells from day 12.5. (G) eGFP-DAZL interaction with RFP-PUM2 in embryonic germ cells from day 12.5. Panels I - VI in each row as follows: (I) A reference image showing expression of eGFP fusion constructs throughout the stem cell. (II) A second digital image obtained from the same plane showing expression of RFP fusion construct. (III) Colocalization of panels I and II depicted in yellow with the location of the nucleus indicated by an arrow. (IV) Phase contrast overlay of Panels I and II. (V) FRET detected by stimulating the cells with blue light and using the acceptor filter for the RFP protein. (VI) FRET/donor ratios as graphed as a function of acceptor/donor ratios.



**Fig. 6.**

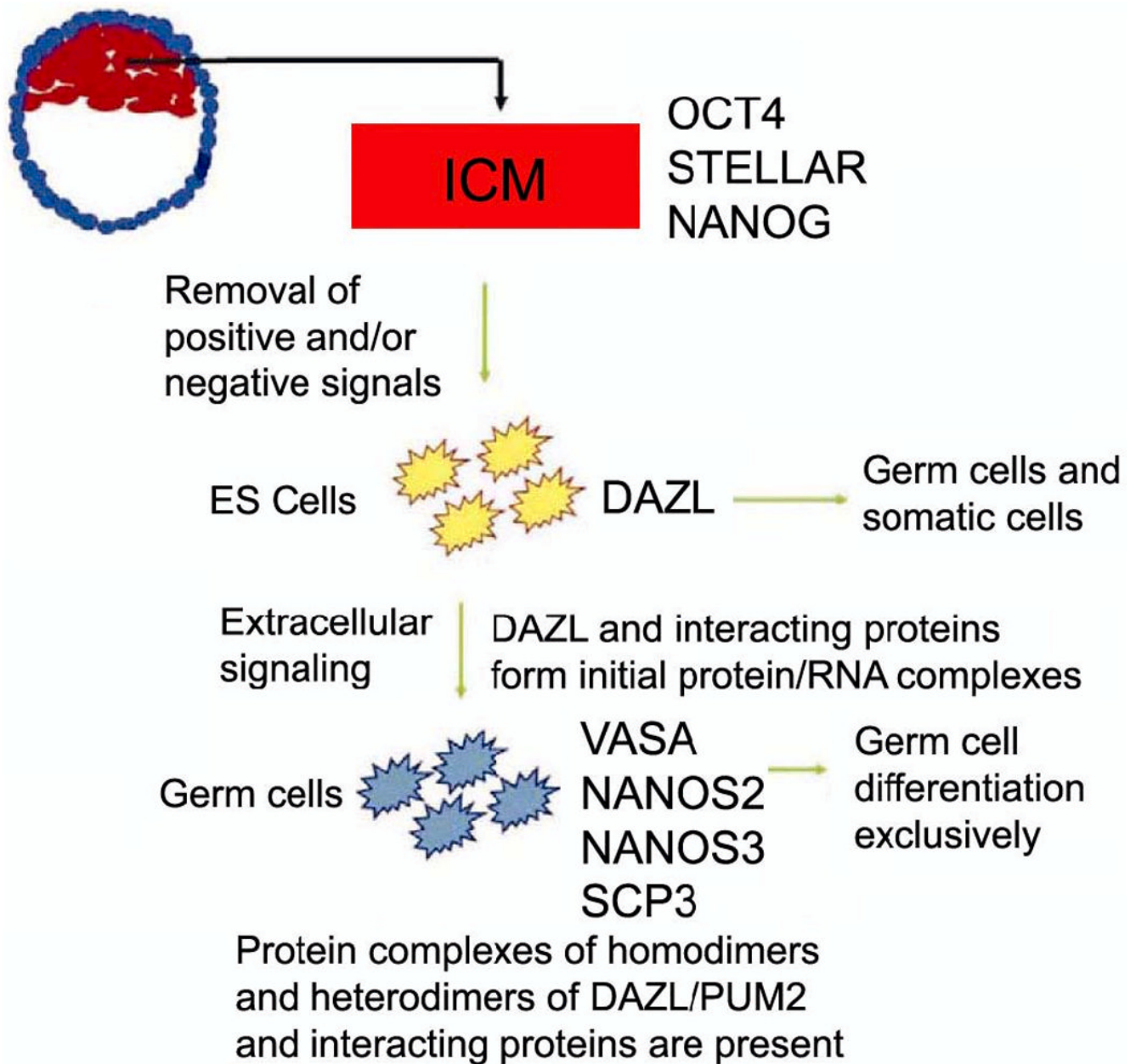
DAZL and PUM2 proteins in living mouse Leydig cells. (A) eGFP-DAZL interaction with RFP-DAZL. (B) eGFP-PUM2 interaction with RFP-PUM2. (C) eGFP-DAZL interaction with RFP-PUM2. Panels I - VI in each row as follows: (I) A reference image showing expression of eGFP fusion constructs throughout the stem cell. (II) A second digital image obtained from the same plane showing expression of RFP fusion construct. (III) Colocalization of panels I and II depicted in yellow. (IV) Phase contrast overlay of Panels I and II. (V) FRET detected by stimulating the cells with blue light and using the acceptor filter for the RFP protein. (VI) FRET/donor ratios as graphed as a function of acceptor/donor ratios.

**Fig. 7.**

Multiple interacting proteins may bind the same RNA. (A) Diagram illustrating known protein-protein interactions with DAZL and PUM2. In addition to interacting with each other, DAZL and PUM2 can interact with several other RNA-binding proteins that also, in turn can interact with each other. (B) Binding of proteins to the 3' UTR of an mRNA transcript with DAZL and PUM2 consensus binding sequences. Fragments are numbered from 0 to 837, corresponding to the stop codon and polyadenylated tail of the *SDAD1* transcript, respectively. Blue color indicates binding of the RNA sequence by the indicated proteins in the yeast three-hybrid assay. Binding was detected for fragments 0-90, 128-218, 332-422, 462-552, 530-620, 666-756 and 734-837 by several proteins, but not DZIP1. (C) Specificity of PUM2 and DAZL binding to

the 3' UTR of *SDAD1*. Using yeast three hybrid and electrophoretic mobility shift (gel shift) assays, PUM2 was shown to recognize five regions within the 3'UTR of the transcript *SDAD1* that were divided into two separate binding elements. The nucleotides required for binding for PUM2 binding element 1 are highlighted in red and a consensus binding element was also defined. Similarly, two regions were mapped for DAZL and a consensus sequence determined by alignment with all four regions bound by DAZL is shown below (Fox et al., 2005).





**Fig. 8.**  
Model of germ cell differentiation based on these studies and others. See text for further explanation.