A Role for *Dazl* in Commitment to Gametogenic Fate in Embryonic Germ Cells of C57BL/6 Mice

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SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

SEPT 2005

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Submitted to the Department of Biology on June, 2005
in Partial Fulfillment of the Requirements for the Degree of
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Abstract

Germ cells can be defined as the cells that undergo the terminal differentiating process of meiosis. In mice, as XX germ cells enter meiosis around Embryonic days 13.5-14.5 (E13.5-E14.5), they form meiotic figures and down-regulate pluripotency markers. XY germ cells enter proliferation arrest between E13.5 and E16.5, which is accompanied by a distinct morphological change as well.

Disruption of mouse \textit{Dazl}, a member of a germ-cell-specific gene family found in many metazoans, causes infertility due to germ cell loss. However the nature and timing of germ cell loss has proven variable in the mixed background mice studied thus far, and the focus has traditionally been on the postnatal spermatogenic phenotype. Here I report a role for \textit{Dazl} in both XX and XY embryonic germ cell development in a C57BL/6 genetic background.

I find that \textit{Dazl} transcript is expressed in the XX and XY gonads starting from around E11.5. XX and XY \textit{Dazl} -/- germ cells appear to arrest around E13.5 in the last cell type XX and XY germ cells share morphologically. In this, they resemble embryonic XX germ cells deficient for \textit{Stra8}, a gene required for meiotic initiation.

Between E14.5 and birth, nearly all XY \textit{Dazl} -/- germ cells die by apoptosis, while the majority of XX \textit{Dazl} -/- germ cells persist through birth. However, XX germ cells require \textit{Dazl} to form meiotic figures, as well as to correctly express \textit{Stra8} and meiotic prophase markers SCP3, \gamma H2AX and \textit{Dmc1}. XX \textit{Dazl} -/- germ cells also fail to down-regulate the pluripotency markers \textit{Oct4} and \textit{Dppa3/Stella}, while XX \textit{Stra8} -/- germ cells down-regulate \textit{Oct4} normally. From this I conclude that exit from pluripotency (as represented by \textit{Oct4} loss) and entry into meiosis (as represented by \textit{Stra8} expression) are at least partially separable processes in XX embryonic germ cells, and that both require \textit{Dazl}. I propose that \textit{Dazl} functions at around E12.5 to allow XX germ cells to exit a pluripotent state, and to acquire competence to undergo meiosis, a fundamental capability that defines germ cells.

I further speculate that \textit{Dazl} is required in early embryonic XX and XY germ cells to acquire differentiation competence- including a capability to exit a pluripotent state, and respond to germ cell sex differentiation signals by entering meiosis or proliferation arrest- and hence commit to a sexually dimorphic, gametogenic fate.

Thesis Supervisor: David C. Page
Title: Professor of Biology, Howard Hughes Medical Institute
For my father, who would have been delighted
Acknowledgements

This work represents the cumulative work, intellectual input and support in every way of a cast of people who cannot even all be named. However, I’d like to specially thank Jana Koubova, Andy Baltus, Yueh-Chiang Hu, Mark Gill, Steve Rozen and Jennifer Hughes for allowing me to refer to work that is either entirely theirs, or the result of collaborations. I would like to thank all the above for intellectually stimulating conversation and also Alex Bortvin. In particular Steve Rozen has been the unnamed mentor of my written work. Richa Saxena was the graduate student whose project I inherited, Steve Wasserman and Tomoko Kaneko are people whose collaborations I do not discuss here, but whose mentorship and help I’ve appreciated a great deal. Mary Goodheart, Laura Brown and Michael Brown are people whose technical expertise permeates my work. Thanks also go to Laura Brown, Julian Lange, Jessica Alfoldi, Jana Koubova, Andy Baltus, Doug Menke, Tomoko Sawai, the entire Page Lab, Mary Lee, Jennifer Hammock, Chenchui Ong, Tracey Ho and others, who have made the years here special for me. Thank you David, for your mentorship and support. You’ve shown me, beyond just the science, what it is to be a gracious scientist.
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Chapter I

Introduction

Murine Germ Cell Specification and Early Development

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Germ line cells are the only immortal cell lineage in metazoans. They alone can directly give rise to subsequent generations. Hence, the capacity to undergo meiosis to give rise to sexually dimorphic gametes (which go on to give rise to the totipotent embryo after fertilization) can be said to define germ line cells in diploid metazoans.

The early development of germ cells has been studied in model organisms such as *Drosophila melanogaster, Xenopus laevis, Caenorhabditis elegans* and *Mus musculus*. The processes that lead to the production of haploid, sexually dimorphic gametes have diverged in fundamental ways across evolutionary time. These processes include (but are not limited to) how germ cells are specified, migrate to and interact with the somatic gonad, switch from mitosis to meiosis and undergo sexual differentiation. For example, in germ cell sex determination, *C. elegans* hermaphrodites have germ line cells that switch from producing sperm to egg, while *Drosophila* and murine germ line cells are committed to a sexual fate by the time they enter meiosis. Germ cell specification itself varies widely across metazoans. *Drosophila, C. elegans* and *Xenopus* germ line cells are specified by maternally inherited factors, while mouse germ cells do not appear to be pre-determined, but are specified by extrinsic signals.

In spite of the obvious differences, there are similarities that can be traced across phyla, and recently it has been revealed that even some molecular components of the processes involved in early germ cell development have been conserved. A theme that dominates early germ cell development is the maintenance of an undifferentiated or stem cell state. In the early embryo, cells are exposed to a variety of signals that induce differentiation down somatic pathways. A variety of processes are employed by germ cells from different species to prevent germ cells from receiving or responding to these
signals. Global transcriptional repression in \textit{C. elegans} and \textit{Drosophila}, specifying germ cells in extra-embryonic regions in vertebrates, using molecular machinery that maintain adult germ line stem cells to prevent primordial germ cell (PGC) differentiation in \textit{Drosophila} and even germ plasm itself, have all been suggested as mechanisms by which early germ cells avoid differentiating down somatic pathways (Deshpande et al., 1999; Dixon, 1994; Gilboa and Lehmann, 2004; Seydoux et al., 1996; Seydoux and Strome, 1999). Eventually germ line cells reverse these processes and the cells commit to a terminally differentiated gametogenic pathway. For instance, in \textit{Drosophila}, transcriptional repression is lifted after pole cells reach the mid-gut and \textit{vasa}, a germ-cell-specific gene, is the first embryonic transcript expressed by pole cells. In mice, germ cells respond to signals from the somatic gonad to express germ-cell-specific markers, including \textit{Mouse Vasa Homolog (Mvh)}. (Toyooka et al., 2000; Van Doren et al., 1998).

Here I review how embryos set aside a population of cells to become future germ cells (germ cell specification) and the processes by which these cells become committed to a germ cell fate. Where possible, I describe common mechanisms and then draw parallels or distinctions between murine germ cells and those of other model species. Of specific interest is a group of genes whose homologs in multiple species act in early germ cell development. Finally, I review what is known in mice about how PGCs commit to the process of becoming sexually dimorphic gametes.
A) Specification of Germ Cell Fate

i) Determinants of Germ Cell specification

In general, germ cells are set aside early in embryogenesis. It is thought that this serves to protect them from inductive signals that specify somatic cell fate to which other lineages in the embryo are exposed (Dixon, 1994). In organisms as diverse as nematodes, insects, and some amphibians, inheritance of maternal factors specifies germ cell fate. These maternal factors are found in structures (variously known as germ plasm, pole plasm, nuage, germinal granules, or chromatoid bodies) within the cytoplasm of the oocyte. Generally germ plasm is characterized by polar granules, electron-dense structures unbound by membranes, often found perinuclearly in close association with mitochondria and rich in mRNAs and proteins (Nieuwkoop and Suminski, 1959; Nieuwkoop and Sutasurya, 1976). In *C. elegans* and *Drosophila* these maternal factors partition asymmetrically as soon as the zygote becomes multicellular. The *C. elegans* zygote divides to give a somatic and a germ line blastomere. *Drosophila* pole cells (primordial germ cells- PGCs) are formed from nuclei that enter pole plasm in the posterior-most portion of the egg. These nuclei are the first in the zygote to cellularize, capturing pole plasm within themselves (Deshpande et al., 2004).

The maternal factors localizing to the germ plasm specify germ line fate. In *Drosophila* it is known that ectopic localization of germ plasm by cytoplasmic transfer or localization of mRNA from a single gene, *oskar*, which is a component of germ plasm, is sufficient to induce the formation of ectopic pole cells (Ephrussi and Lehmann, 1992; Illmensee and Mahowald, 1974). Furthermore, once pole cells are formed, they lose their
ability to give rise to somatic lineages (Technau, 1987; Underwood et al., 1980). Hence, at least in *Drosophila*, germ plasm appears to be the germ line determinant.

In other organisms, germ plasm appears to permit specification of rather than determine germ cell fate. In *Xenopus*, injection of germ plasm can rescue the loss of PGCs induced by low level UV irradiation of eggs. However, injecting germ plasm into ectopic locations in the oocyte does not give rise to ectopic germ cells as it does in *Drosophila* (Smith, 1966; Wakahara, 1977). Indeed, transplanted germ plasm can contribute to somatic lineages from all three germ layers— which are the three major cell lineages established during gastrulation, the ectoderm, mesoderm and endoderm (Wylie et al., 1985). These results imply that although the germ plasm is permissive to germ cell fate determination in *Xenopus*, it does not necessarily restrict cells to a germ cell fate, and inductive signals also play a role in germ cell specification.

The molecular mechanism whereby germ plasm specifies, or permits the specification of, germ cell fate is not entirely understood. However, to some extent, germ plasm appears to function via transcriptional repression. Transcriptional repression has been observed in *Drosophila* pole cells and *C. elegans* germ line blastomeres (the germ line cells in *C. elegans* embryos). Somatic blastomeres in *C. elegans* produce new transcripts from the four cell stage onward, but the germ line blastomeres become transcriptionally active only at the 100 cell stage (Seydoux and Dunn, 1997; Seydoux and Fire, 1994; Seydoux et al., 1996). *Drosophila* somatic nuclei activate transcription even before cellularization takes place in the zygote. However, pole cells down-regulate transcription from RNA Polymerase II until pole cell migration to the somatic gonad, well after cellularization (Van Doren et al., 1998).
Transcriptional repression is linked to the maintenance of germ cell identity. In particular, *C. elegans* mutants in *pie-1* (a gene required for transcriptional repression and germ cell maintenance) prematurely activate transcription in the germ line blastomere, which subsequently joins the lineage fate of its sister somatic blastomeres (Mello et al., 1992; Seydoux et al., 1996). This suggests a causal relationship between transcriptional repression and germ cell identity, but also implies that the preservation of germ cell fate is an active process. Similarly, *germ-cell-less*, *polar granule component*, *nanos* and *pumilio* are examples of genes required for both transcriptional repression as well as germ cell maintenance in *Drosophila* (Asaoka-Taguchi et al., 1999; Deshpande et al., 2004; Deshpande et al., 1999). Epigenetic changes in chromatin structure also play a role in transcriptional repression in germ line cells. While *C. elegans* germ line blastomeres rely on Pie-1 protein to initiate transcriptional repression, as Pie-1 degrades, these blastomeres employ special chromatin structures to maintain transcriptional repression. *Drosophila* pole cell nuclei have chromatin in a transcriptionally inactive state even prior to cellularization and maintain this until pole cells migrate to the somatic gonad (Deshpande et al., 2004; Leatherman et al., 2002; Schaner et al., 2003).

i) Murine Germ Cell Specification

Unlike *Drosophila* and *C. elegans* germ line cells, there has been no clear morphological evidence for the existence of germ plasm in mouse oocytes. Although a recent electron microscopic examination of a germ-cell-specific transcript-- *Mvh*-- has revealed that it is localized in the chromatoid body, a perinuclear, electron dense structure in maturing sperm that resembles germ plasm in other species. However, the biological
significance of this structure is unknown (Toyooka et al., 2000). At the same time, much evidence points to murine germ cell specification relying on inductive signals rather than inherited factors.

Murine germ cells are thought to arise soon after gastrulation from a pluripotent cell population in the proximal epiblast. Cell lineage studies show that extraembryonic mesoderm and PGCs arise from the same pool of proximal epiblast cells in early embryos (Lawson and Hage, 1994). Although distal epiblast cells in embryos never form PGCs, transplanting the distal cells into the proximal region can cause these cells to give rise to PGCs, while proximal cells transplanted to the distal region never give rise to PGCs (Tam and Zhou, 1996). These findings imply that location, and not strictly origin, dictates germ cell fate. A series of elegant experiments pinpointed BMP4/8b signaling as being necessary to give rise to PGCs \textit{in vivo} and sufficient to induce epiblast cells to maintain Oct4- and alkaline-phosphatase (AP) cells, i.e. germ-cell-like cells, \textit{in vitro} (Lawson et al., 1999; Ying et al., 2000; Ying et al., 2001). However it has since been shown that although epiblast cells exposed to BMP4/8b signaling can be induced to maintain Oct4 and AP expression, and up-regulate RNA levels of a marker of germ cell competence, \textit{Fragillis}, they do not express the first germ-cell specific marker, \textit{Dppa3/Stella}. Based on these results, other, as yet unknown, signals are postulated to be required for actual germ cell specification (Saitou et al., 2003).

Transcriptional quiescence in mouse germ cells has not been documented. However, expression studies do show that while somatic cells surrounding newly formed PGCs in mice up-regulate levels of somatic markers (among them \textit{Hox} genes), newly formed murine PGCs repress the expression of these genes (Saitou et al., 2003).
Consistent with the lack of evidence of transcriptional repression, and in contrast to *C. elegans* and *Drosophila*, mouse germ cell chromatin undergoes a dynamic series of active and repressive states just after germ cell specification (Seki et al., 2005). It is believed that these changes reflect complex epigenetic regulation, including the acquisition of parental imprinting, which distinguishes mice from worms and flies. It has been speculated that recent successes in deriving germ cells *in vitro* from ES cells have nevertheless not been successful at producing progeny due to the lack of proper epigenetic reprogramming (Geijsen et al., 2004; Hubner et al., 2003; Seki et al., 2005; Toyooka et al., 2003).

Overall, the mechanisms that mice employ to specify and maintain germ cell identity are not as well understood as in *C. elegans* or *Drosophila*, but already it appears that some fundamental disparities exist in terms of induced, compared to preformed, or a combination of induced and preformed germ cell specification, and the global methylation and transcriptional state of early germ cells.

**B) Genes required for early germ cell development across metazoans**

Given the fact that germ cell specification and the epigenetic characteristics of early germ cells are so different in murine germ cells compared to those of other model organisms, it is surprising that many of the genes required for early germ cell development are conserved. Probably the best conserved theme in early germ line development is the maintenance of an undifferentiated/pluripotent state. Whether it is by transcriptional silencing or by maintaining stem cell characteristics, early germ cells across phyla share the common goal of avoiding differentiation down somatic lineages at
a time when the embryo is awash in differentiation signals. Further on in development, these processes that maintain the PGCs undifferentiated state are then reversed or removed and processes that lead the PGC to terminal differentiation as a gamete become important.

Although maintenance of an undifferentiated state, and then commitment to a terminal differentiating process of germ cell differentiation is conserved in PGC development across phyla, the mechanisms through which these processes are accomplished vary. Hence, it is somewhat surprising the extent to which the genes involved in some of these processes are conserved. In particular, I will more closely examine four of these widely conserved gene families with early germ cell functions: Nanos, Pumilio, Vasa, and DAZ. Nanos and Pumilio homologs often interact and function together to specify and preserve PGC identity and an undifferentiated cell state in PGCs, and also in germ line stem cells (GSCs) in some species. Vasa and DAZ are germ-cell-specific across all phyla examined. Vasa homologs are found in the germ plasm of all species with germ plasm equivalents, but have been implicated in germ cell specification only in Drosophila. In other species germ cell differentiation appears to be their earliest function. DAZ homologs have also been found in germ plasm in various species, but have been implicated only in germ cell differentiation albeit at multiple stages.

i) Nanos

nanos was first identified in Drosophila as a maternal gene involved in posterior patterning of the embryo. This function is dependent upon nanos-mediated translational
repression of yet another maternal posterior group gene, *hunchback* (Hulskamp et al., 1989; Irish et al., 1989; Nusslein-Volhard et al., 1987). *Nanos* family genes are identified almost entirely by homology in a zinc-finger motif that, at least in *Drosophila*, is essential to its known functions (Arrizabalaga and Lehmann, 1999).

*Nanos* family genes have been shown to function across multiple vertebrates and invertebrates in germ cell fate maintenance and early PGC development. Maternal *Nanos*-homologues are components of pole plasm in *Drosophila* and *Xenopus* (*Xcat2*). In *C. elegans*, *Nanos*-homologues nos-1 and -2 are involved in germ cell developmental events (MacArthur et al., 1999; Subramaniam and Seydoux, 1999; Wang and Lehmann, 1991). In mice, disruption of *Nos2* and *Nos3* gives fully viable but infertile mice (Tsuda et al., 2003).

In *Drosophila*, maternal *nanos* plays a key role in preventing mitosis, maintaining transcriptional silence, and suppressing somatic fate in early germ line cells. Loss of maternal *nanos* results in pole cells that migrate poorly, are unable to establish or maintain transcriptional quiescence, express somatic genes ectopically, fail to attenuate their cell cycles and undergo apoptosis (Deshpande et al., 1999; Kobayashi et al., 1996). *nanos'* role in transcriptional repression is two-fold: It is required for pole cells to decrease RNA polymerase II activity and also required to maintain a chromatin architecture that is correlated to transcriptional quiescence (Schaner et al., 2003). More recently, in an effort to study *nanos*-deleted pole cells, which usually die by apoptosis during migration, a deletion that removes apoptosis gene *Reaper* was crossed into a *nanos*-deleted background. These *nos-H99* pole cells surprisingly recover migration capability, but also display the ability to take on somatic cell fates. Some of the germ
cells lose expression of germ-cell-specific marker vasa and gain markers of somatic fate. This implies that nanos plays a role in the maintenance of germ cell fate, but also implies that germ cells remain somewhat pluripotent, at least during migration (Hayashi et. al., 2004). This link between pluripotency and germ cell fate maintenance will be revisited when I discuss the fact that, like nanos, pumilio too is required for both processes [see page 19, and (Gilboa and Lehmann, 2004)]

While maternal nanos in Drosophila acts to maintain germ cell identity in PGCs by suppressing somatic fate, zygotic nanos is involved in the maintenance of GSCs in the ovary. Flies transheterozygous for strong nanos-deficiency alleles often produce a small number of eggs early in life before becoming entirely infertile, and flies which have nanos removed only from their GSCs show a single wave of differentiation into egg chambers (Forbes and Lehmann, 1998; Wang and Lin, 2004). These data imply that at least one of nanos’ functions is to enable GSC self-renewal.

Biochemically, nanos is known to function together with RNA-binding protein pumilio as a translational repressor, for example of hunchback. pumilio and nanos together translationally repress cyclinB to attenuate cell cycle in migrating germ cells. The repression by pumilio and nanos of cyclinB is dependent on a Nanos Response Element-like (NRE-like) sequence on the 3’ UTR of cyclinB mRNA (Asaoka-Taguchi et al., 1999).

There are three C. elegans nanos homologs. nos-1 is required for efficient entry of germ cells into the somatic gonad, nos-2 maintains germ cell viability in the larva, and also prevents germ cells from ectopic divisions in the starved animal, while nos-3 is involved in the control of mitosis versus meiosis as well as sperm versus oocyte
production in hermaphrodites (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). In the control of the proliferation-to-meiotic-differentiation switch, nos-3 acts genetically either in parallel or downstream of fbfl/2 (C. elegans Pumilio homologs) to regulate levels of GLD-1 protein. High levels of GLD-1 promote meiotic entry and low levels of GLD-1 allow mitosis (Hansen et al., 2004). NOS-3 protein also physically interacts with FBF-1/2, which were first identified as repressors of fem-3, a gene that regulates the spermatocyte-to-oocyte switch (Zhang et al., 1997). When nos-1 or 2 are knocked down in a nos-3 mutant background, some hermaphrodites fail to switch to oogenesis, producing an excess of sperm instead. This is taken as evidence that nos-3 plays a role in the spermatocyte-oocyte switch- though some redundancy with nos-1/2 and other unknown factors is evident (Kraemer et al., 1999).

Vertebrate Nanos homologs have more recently been discovered and studied. The Xenopus protein related to Drosophila Nanos, known as Xcat2, has been shown to bind RNA in vitro and physically interact with Xenopus Pumilio protein to regulate CyclinB1 translation (MacArthur et al., 1999; Nakahata et al., 2001; Nakahata et al., 2003). There are three Nanos homologs in mice. Nanos1 is expressed primarily in the central nervous system, and despite some expression in adult ovaries and testes, mice deficient for Nanos1 are viable and fertile with no apparent defects (Haraguchi et al., 2003). The expression pattern of Nanos2 is very narrow. It is only expressed in XY germ cells between embryonic days 13.5 (E13.5) and E16.5. Nanos2 knockout mice show germ cell loss by apoptosis and mislocalization starting from E15.5, with complete loss by 4 weeks after birth. Nanos3 is expressed in both XX and XY germ cells at least from E9.5, but is down-regulated by E14.5 in XX germ cells and E15.5 in XY germ cells before becoming
up-regulated again in XY gonads a few days after birth. This is a pattern that coincides with the presence of mitotically active germ cells in both genders. Both XX and XY \textit{Nanos3} knockout mice have no germ cells by E12.5, although germ cell numbers seem normal at E7.5 and no signs of apoptosis accompanies germ cell loss (Tsuda et al., 2003). Human \textit{NANOS} has recently been discovered, \textit{NOS1}, and was found to physically interact with one of two human \textit{PUMILIO} homologs, \textit{PUM2}. Both \textit{PUM2} and \textit{NOS1} are highly expressed in spermatogonia and early spermatocytes (Jaruzelska et al., 2003).

It appears that the various \textit{Nanos} genes in \textit{C. elegans} and \textit{Nanos} in \textit{Drosophila} serve a set of functions that overlap to a large extent: attenuation of mitosis, PGC survival, germ cell migration, and regulation of the GSC-to-egg-chamber/mitosis-to-meiosis switch. While vertebrate \textit{Nanos} homologs have only just begun to be studied, there is clear evidence that the physical interaction of \textit{Nanos} with \textit{Pumilio} has been conserved even in mammals. There is even some evidence of conservation of function between vertebrates and invertebrates, as with the translational repression of \textit{CyclinB} in \textit{Xenopus} and \textit{Drosophila} (Nakahata et al., 2001).

\textit{ii) Pumilio and Fbf (Puf)}

\textit{Drosophila pumilio} and the \textit{C. elegans fbf} genes (Puf) are the founding members of a family of RNA-binding proteins characterized by the Puf domain- eight tandem RNA-binding repeats (Zamore et al., 1997). There are Puf family proteins in organisms ranging from yeast to planarians to humans, and it is thought that they have an ancestral, partially conserved function in the decision between stem cell maintenance and differentiation (Crittenden et al., 2002). Indeed it has recently been found that planarians
(Dugesia japonica) also have a homolog of Pumilio, DjPum, which is expressed highly in neoblasts— the stem cell population which give planarians their remarkable regenerative abilities. Knocking down DjPum using RNAi reduces the numbers of neoblasts dramatically (Salvetti et al., 2005). Further, the levels of Saccharomyces cerevisiae UTH4 protein, a Puf family member, correlate to the lifespan potential of yeast cells. Increasing UTH4 levels can extend the number of daughter cells that mother cells can produce before dying (Kennedy et al., 1997).

*Drosophila pumilio*, like *nanos*, was first discovered as a maternal gene involved in the posterior patterning of *Drosophila* embryos. *Drosophila* Pumilio protein specifically binds Nanos Response Elements (NREs) on target mRNAs, and is thought to then recruit Nanos by protein-protein interaction to act as translational repressor (Murata and Wharton, 1995). *pumilio* and *nanos* together have also been shown to repress translation from an internal ribosome entry site (IRES) (Wharton et al., 1998).

Maternal *Drosophila pumilio* acts with *nanos* to regulate transcriptional quiescence, mitotic attenuation and proper migration in pole cells (Asaoka-Taguchi et al., 1999). In the absence of pumilio, pole cells prematurely express a germ line marker, undergo mitosis ectopically while migrating and migrate aberrantly—much the same phenotype as the lack of *nanos* produces. Specifically, *Drosophila* pole cells maintain a G2 mitotic quiescence during their migration but in the absence of *pumilio* or *nanos*, pole cells suffer an early release from G2 arrest, implying that *pumilio* and *nanos* are required for the repression of G2/M transition in these germ cells (Asaoka-Taguchi et al., 1999).

Embryonic *Drosophila pumilio* is required for asymmetric division and maintenance of germ line stem cells in the ovary (Lin and Spradling, 1997). A novel
class of pumilio mutations were discovered that gave rise to females which were infertile due to a loss of asymmetric division that maintain the germ line stem cells—instead all the stem cells differentiated in a symmetric division to give a very small number of egg chambers (Lin and Spradling, 1997).

From these experiments it was observed that both maternal and embryonic pumilio are required to suppress premature differentiation in germ cells, albeit at different stages of embryonic development. Indeed, it was noted that PGCs and germ line stem cells (GSCs) employ much the same molecular machinery to maintain an undifferentiated state, a mechanism that includes, but is not limited to nanos and pumilio. It was further suggested that PGCs and GSCs are not so different and that the larval gonad is the equivalent of the stem cell niche in adult ovaries (Gilboa and Lehmann, 2004).

In C. elegans, FBF-1 and FBF-2 proteins promote mitosis and self-renewal of germ line stem cells in hemaphrodites by repressing the translation of gld-1, a meiosis-promoting translational regulator. This regulation is very tightly controlled, and the number of mitotically proliferating germ cells within the hermaphrodite gonad can be regulated by the dosage of the fbf-1/2 genes. Single mutants (fbd-1 or fbd-2) have a smaller mitotic region (fewer mitotically active germ cells) than wildtype worms but larger mitotic regions than the fbf-1/2 double mutant (Crittenden et al., 2003). FBF-1/2 were initially isolated as proteins that bound the 3’ UTR of the C. elegans sex-determination gene fem-3 which regulates the sperm-to-oocyte switch in hemaphrodites. They have since been shown, along with C. elegans NANOS3, to regulate fem-3 postranscriptionally (Kraemer et al., 1999; Zhang et al., 1997). There are at least four other Puf family genes in C. elegans, puf-6, 7, 8 and 10. Disruption of various
combinations of these genes along with disruptions in \textit{fbf}-1/2 give rise to various PGC defects, ranging from migration defects to germ cell death. This suggests that there are multiple, at least partially redundant, interactions between various \textit{nanos} and \textit{pumilio} homologs in \textit{C. elegans}.

However, not all members of the Puf family in \textit{C. elegans} promote stem cell maintenance over differentiation as the examples cited here suggest. \textit{C. elegans} PUF8 promotes meiotic differentiation and primary spermatocytes lacking PUF8 dedifferentiate into mitotically active germ cells and give rise to germ cell tumors (Subramaniam and Seydoux, 2003).

\textit{CyclinB1} translational repression is a function of \textit{Xenopus Pumilio} (\textit{Xpum}). XPUM protein has been shown to physically interact with the NRE of \textit{Drosophila} \textit{hunchback} and the 3’ UTR of \textit{Xenopus CyclinB1}. It also interacts with \textit{Xcat2} protein (\textit{Nanos} homolog in \textit{Xenopus}) and unphosphorylated cytoplasmic polyadenylation elements binding protein (CPEB)- which also binds the 3’ UTR of \textit{CyclinB1} (Nakahata et al., 2001). Injection of anti-XPUM into oocytes increases \textit{CyclinB1} translation and oocyte maturation, while an increase in \textit{Xpum} levels does the converse. Hence \textit{CyclinB1} is a target for Xpum translational repression in \textit{Xenopus} oocytes (Nakahata et al., 2003). It is postulated that the phosphorylation of CPEB and Xpum’s subsequent dissociation from CPEB and \textit{CyclinB1} mRNA is what allows translation of \textit{CyclinB1} mRNA to proceed (Nakahata et al., 2003).

Murine \textit{Pum1} and \textit{Pum2} are expressed in a wide variety of cells, and PUM2 protein interacts with the mRNA of mitosis regulating protein \textit{Proliferation potential protein-related} (\textit{P2P-R}), raising the possibility that the regulation of mitosis is a function
of at least one mammalian Pumilio (Spassov and Jurecic, 2003). Pum2 has a consensus binding site distinct from NRE, called Pum2 binding element (PBE). Most recently, PBEs were found in murine P2P-R’s 3’ UTR, and the P2P-R mRNA found to physically interact with PUM2 protein (Scott et al., 2005). Murine P2P-R is a nuclear protein highly upregulated in mitotic cells whose function was speculated to be the promotion of mitotic proliferation (Gao et al., 2002). However, over-expression of P2P-R in cultured cells resulted in an unexpected cell cycle arrest in mitotic prometaphase (Gao and Scott, 2002).

Humans have two Pumilio homologs, PUM1 and PUM2, which are widely expressed. Like Drosophila pumilio, human PUM2 binds to NREs (Fox et al., 2005; Jaruzelska et al., 2003). Recently, human PUM2 protein has been found to bind to all three DAZ family proteins found in humans- DAZ, DAZL and BOULE (Moore et al., 2003; Urano et al., 2005). The DAZ family proteins are all also RNA-binding proteins (see page 24 for more about DAZ family members) and this interaction has been interpreted to mean that DAZ family members may regulate some of the same mRNA partners that PUM2 does in humans. Hence, RNA targets of human PUM2 and DAZL have been isolated from human testis mRNA pools. Of 61 candidate binding partners of both PUM2 and DAZL, SDAD-1 was singled out for further mention in the publication (Fox et al., 2005). SDAD-1 is a homolog of a S. cerevisiae gene, sda-1, that is required for passage through G1, likely due to its actin cytoskeleton organization activity (Buscemi et al., 2000; Zimmerman and Kellogg, 2001). The possibility that PUM2 and DAZL interact to regulate cell cycle control in human germ cells was raised.

Overall, cell cycle control (or a stem cell/mitotic fate versus differentiation/non-mitotic fate decision), particularly in germ cells, appears to be a function of Puf family
genes across phyla. *Drosophila* and *C. elegans* Puf family genes also have functions in PGC migration and survival. However, the presence of multiple Puf family members in many species (particularly *C. elegans*) and the fact that genetic deficiencies of Puf family genes in vertebrates have not been studied makes it more difficult to define what the conserved functions of Puf family genes are.

iii) Vasa

*Vasa* family genes are DEAD box RNA helicases which are a large family of proteins involved in unwinding RNA-RNA or RNA-DNA duplexes, and have been known to be involved in regulating RNA stability, processing, editing, export from the nucleus, and translation (de la Cruz et al., 1999; Luking et al., 1998). *Drosophila vasa*, the founding member of the *Vasa* family, localizes to the pole plasm in an *oskar*-dependent manner, and is essential for pole plasm assembly. Indeed, Vasa protein interacts directly with Oskar protein, and this interaction is required for germ plasm assembly (Breitwieser et al., 1996). *vasa* and *oskar* are required for the localization of *nanos* mRNA to the pole plasm, and *vasa* itself functions to upregulate translation of some mRNAs found in germ plasm, amongst them *nanos* and *oskar* mRNA (Gavis et al., 1996; Tomancak et al., 1998).

*Vasa* homologs have been reported as components of germ plasm in *Xenopus*, zebrafish and chickens, as well as p-granules (germ-plasm-like structures) in *C. elegans* (Bilinski et al., 2004; Gruidl et al., 1996; Knaut et al., 2000; Tsunekawa et al., 2000). Even in mice, which have not been observed to have germ plasm, *Mvh* is reported to
localize to chromatoid bodies in spermatid (Toyooka et al., 2000). Chromatoid bodies are structures that resemble germ plasm in other organisms.

*C. elegans* has at least four members of the *GLH* family (homologs to *Vasa*), and RNAi knockdown of *glh-1/4* in hermaphrodites results in offspring that lack oocytes and have defective sperm. However, p-granules (components of *C. elegans* germ plasm) can still be observed and hence *glh-1/4* are not thought to be required for germ cell specification, but instead for germ cell development (Kuznicki et al., 2000).

*Xenopus* Vasa-like gene (*XVLG-1*) is co-expressed with *Xcat2* (*Nanos* homolog) in germ plasm. However, from injections of anti-*XVLG-1* into early embryos, *XVLG-1* appears to be required for PGC differentiation at the tadpole stage, but not germ cell specification (Ikenishi and Tanaka, 1997). Injections of *XVLG-1* were not sufficient to produce supernumerary PGCs, indicating that *XVLG-1* alone is not a determinant of PGC fate (Ikenishi and Yamakita, 2003).

In zebrafish, *vas* (*Vasa* homolog) mRNA localizes to the germ plasm, although maternal protein is observed throughout the embryo (Braat et al., 2000; Knaut et al., 2002). A *vas:EGFP* construct also showed an unexpected difference in splicing of *vas* between male and female zebrafish germ cells. This result is interesting particularly because so little is known about determinants of sexual differentiation in zebrafish (Krovel and Olsen, 2004). Knockdown of zebrafish *vas* at the single cell stage suggests that zygotic translation of *vas* is not required at least within the first four days after fertilization (beyond which *vas* mRNA levels recover) as no PGC phenotype is observed.

Many of the genes that localize to the germ plasm in other species are first observed in murine germ cells when they arrive at the somatic gonad (genital ridge).
Mvh (also known as Ddx4), along with Nanos2 and Dazl, is one such gene. Mvh expression is germ cell specific and its expression can be induced in cultured embryonic germ cells after exposure to somatic gonadal cells (Toyooka et al., 2000). Mvh is required only in male germ cell development as Mvh knockout females are fertile. In male mice, loss of Mvh causes germ cells to mislocalize outside the cords and germ cell loss at E12.5. The embryonic germ cell loss is not complete and some germ cells progress to zygotene of the first meiotic division in the postnatal testis before dying by apoptosis (Tanaka et al., 2000). The male Mvh knockout phenotype is reminiscent of the male Dazl knockout phenotype (Ruggiu et al., 1997).

Although Vasa homologs are widely used to identify germ cells as their expression has been found to be germ-cell-specific across many species (gibel carp and sea anemone being the latest species), very little is understood about how these RNA helicases function outside of Drosophila. In Drosophila, vasa is required for pole plasm assembly and hence germ cell specification, dependent upon protein-protein interactions and translational regulation. In Xenopus, C. elegans and mice, Vasa homologs are not to be required for germ cell specification, but rather early germ cell development, although no mechanisms for their function have been discovered.

iv) DAZ

The DAZ proteins contain a highly conserved RNA-recognition motif (RRM) and have germ cell specific expression and function in metazoans. The founding members of the DAZ family were found on the human Y chromosome in a region whose deletion leads to an absence of sperm in human males (azoospermia), hence the name Deleted in
Azoospermia (DAZ) (Reijo et al., 1996). Y-linked DAZ are found only in the Old World Monkey lineage. Other vertebrates are though to have only the two autosomal homologues, Dazl (for DAZ-like) and Boule, while non-vertebrates have only one family member, autosomal Boule. By sequence homology, it appears that Dazl and Boule arose from a duplication in the vertebrate lineage, and DAZ arose from a copy of DAZL transposed to the Y chromosome in the Old World Monkey lineage (Xu et al., 2001).

In Y-linked DAZ genes, the exons that code for the RRM are intragenically duplicated up to three times, and the exon that codes for the DAZ repeat are similarly duplicated up to 17 times (Saxena et al., 2000). The DAZ gene itself has been duplicated twice to give a total of 4 copies on most human Y chromosomes. It is not known what functional significance, if any, these duplications have. However, it is worth noting that the duplication that gave rise to the 4 copies of DAZ in most modern humans and chimpanzees most likely occurred independently in both the human and the chimpanzee lineage (Steve Rozen, Jennifer Hughes; personal communication). This suggests that having 4 rather than 2 DAZ copies might confer some sort of selective advantage to those Y chromosomes; enough to have carried them to fixation in both lineages.

All known disruptions of DAZ family genes lead to male and/or female infertility due to germ cell loss (Eberhart et al., 1996; Hashimoto et al., 2004; Houston and King, 2000; Karashima et al., 2000; Reijo et al., 1995; Ruggiu et al., 1997; Xu et al., 2001). The range of phenotypes, however, is quite broad, with some sex specific effects even in the autosomal family members. While Drosophila Boule is required for progression through G2-M only in male flies, loss of daz-1 in C. elegans leads to meiotic prophase arrest only in oogenesis (Karashima et al., 2000; Maines and Wasserman, 1999).
The meiotic defect in *C. elegans* was thought to be the earliest phenotype of the *daz-1* deficiency. However, a more recent study showed that the protein is most highly expressed in mitotic oogonia just prior to meiosis entry, and that early *daz-1* oocytes have subtle morphological defects. This has led to speculation that *daz-1* is actually involved in germ cell differentiation events prior to meiotic prophase and perhaps prior to meiosis entirely (Maruyama et al., 2005).

The testes of loss-of-function *boule* homozygous flies are filled with 16-cell cysts that are phosphohistone H3 negative and lack meiotic spindles—indicating that the first meiotic division has not taken place and the G2-M transition has not been made. This is very similar to *twine* (*Drosophila* meiosis-specific Cdc25 phosphatase) loss of function homozygous flies. In a *boule* loss-of-function background, protein expression from *twine-lacZ* reporter construct is dramatically lower than in wildtype testis while *twine-lacZ* mRNA accumulates. A construct with the *twine* ORF inserted between the 5’ and 3’ UTRs of a meiosis-specific tubulin, which can be translated independently of *boule*, can rescue the *boule* mutant’s meiotic entry defect (Maines and Wasserman, 1999). From this finding it can be concluded that *boule* functions to promote the G2-M transition in meiosis I of spermatogenesis by up-regulating *twine* translation. Whether *boule* does so by directly binding *twine* mRNA is not known.

*Xenopus Dazl* homolog (*Xdazl*) and zebrafish *Dazl* homolog (*zDazl*) mRNA can be found in maternally derived germ plasm (Houston et al., 1998; Maegawa et al., 1999). Depletion of maternal *Xdazl* in tadpoles early in development results in later stage PGCs losing the ability to migrate and rapidly being lost. *Xdazl* is hence thought to be required
for some early step in PGC differentiation that confers "migration competence" (Houston and King, 2000).

From the expression and mutant studies in various organisms it has been suggested that the Boule genes are involved in meiotic progression, while the Dazl genes are involved earlier in germ cell development. However, it has been shown that Xdazl can rescue the meiosis prophase I arrest phenotype in Boule mutant flies, while zDazl can activate translation of Drosophila twine in vivo. Hence, the separation of function ascribed to the sub-classes of DAZ family proteins is incomplete (Houston et al., 1998; Maegawa et al., 2002).

Translational regulation is thought to be the conserved function of DAZ family proteins. Several lines of evidence have led to this assumption: First, the RRM’s are highly conserved between family members, from worms through humans, and have been shown to bind RNA’s in vitro (Jiao et al., 2002; Venables et al., 2001). Secondly, Drosophila boule induces progression through G2-M of the first meiotic prophase via up-regulating translation of meiosis-specific phosphatase twine (Maines and Wasserman, 1999). Third, Zdazl has been shown in some elegant co-transfection studies to activate translation of Drosophila twine and Zdazl itself in a manner dependent on (GUUC)n sequences in the genes’ 3’ UTR’s (Maegawa et al., 2002). And last, murine Dazl protein has been shown to colocalize with ribosomes in sedimentation experiments (Tsui et al., 2000). Thus far all evidence points to translational regulation as a conserved function of DAZ family proteins.

The assumption that the DAZ family proteins are translational regulators has led to a search for RNA substrates that DAZ family proteins bind. Two such studies have
looked for potential RNA targets of the murine Dazl protein, have found that mouse
DAZL binds U-rich sequences in the 3’ UTR of potential targets, including Cdc25A and
Cdc25C (two of the three murine homologs of Drosophila twine), Tpx-1 (a gene thought
to function in germ cell-Sertoli cell interaction) and Trf2 (a gene required for
spermiogenesis). The biological significance of these targets is not known. Indeed, some
of these genes are not expressed until after germ cells die in knockout Dazl mice (Jiao et
al., 2002; Venables et al., 2001).

Murine Dazl protein is found in PGCs and oocytes as well as spermatogonia and
early spermatocytes, with the highest level of expression occurring in pachytene
spermatocytes (Ruggiu et al., 1997). Murine Boule protein is not expressed in PGCs nor
in spermatogonia but is instead first observed in spermatocytes and can be found in all
stages of spermatogenesis from spermatocytes up through early spermatids. Boule’s later
expression pattern and its closer sequence similarity to Drosophila boule compared to
Dazl in other species has led to the speculation that murine Boule may be involved in
meiotic progression while Dazl’s earlier expression may indicate an earlier premeiotic
function (Xu et al., 2001). Indeed the earliest zygotic murine Dazl transcript is observed
at E11.5, as assayed by RT-PCR, just prior to the time germ cells undergo sexual
differentiation (Seligman and Page, 1998).

Previous studies, all conducted in mixed genetic backgrounds, suggest that Dazl is
essential to XY germ cell development and survival only later in development, although
reported time frames varied (Maratou et al., 2004; Ruggiu et al., 1997; Saunders et al.,
2003; Schrans-Stassen et al., 2001). In the initial study reporting the knockout (see Fig. 1
for a diagram of the knockout loci) both XX and XY E15.5 germ cells are reported to be
normal in appearance and germ cell numbers. At this time point, XX \textit{Dazl} -/- germ cells are in the first meiotic prophase and XY \textit{Dazl} -/- germ cells do not present obvious morphological problems as they enter mitotic arrest. The earliest phenotype is at E19.5, when a drop in germ cell to Sertoli cell ratio in the XY gonads and a severe loss of XX germ cells is observed. XX germ cells almost all die embryonically while some XY germ cells survive to enter meiosis postnatally (Ruggiu et al., 1997). A subsequent publication characterized the XY \textit{Dazl} -/- phenotype as a failure of postnatal spermatogonial differentiation, with no significant difference between germ cells of wildtype and \textit{Dazl} -/- testes until nine days after birth (D9). The BrdU labeling index, the proportion of c-kit positive germ cells and morphology of germ cells from testis of \textit{Dazl} -/- adult males were used to judge that the arrest in \textit{Dazl} -/- germ cells occurred at a stage of spermatogonial development similar to the arrest observed in Vitamin-A deficient (VAD) mice (Schrans-Stassen et al., 2001). A third publication found that the latest defect in XY \textit{Dazl} -/- germ cell development is a block in the leptotene-zygotene transition in the first wave of meiosis, occurring one to two weeks after birth. The spermatogonial defect seen in the \textit{Dazl} -/- adult testis from the previous paper was interpreted as the terminal phenotype. While they saw varying numbers of germ cells as well as abnormal, multinucleate germ cells at D2 and D4 in the \textit{Dazl} -/- testes, germ cell loss was consistently observed only at D7 and beyond. D19 \textit{Dazl} -/- testes were examined for markers of meiosis, and cells positive for \textit{yH2AX} and cells positive for SCP3 (both markers of leptotene spermatocytes) were found but no cells were found that were positive for H1t (a marker of zygotene spermatocytes).
Fig. 1: Diagram of the wildtype and *Dazl*<sup>TM1/Hgu</sup> genetic loci. The Neo cassette replaces exons 6 and 7 and part of exon 5 in the *Dazl*<sup>TM1/Hgu</sup> construct, removing part of the RRM and the entire DAZ repeat.
Wildtype Dazl loci (partial)

Exons

Dazl<sup>TM1/Rigu</sup> allele (partial)

Exons

- RNA recognition motif (RRM)
- DAZ repeat
- Neo cassette
XX Dazl -/- germ cells were examined as well, and found, by morphology, to enter pachytene normally, but die prenatally (Saunders et al., 2003). Most recently, a study comparing the RNA expression profiles of postnatal XY Dazl -/- and wildtype testes also concluded that leptotene-zygotene of meiosis is the most advanced stage attained by XY Dazl -/- germ cells (Maratou et al., 2004).

Overall, DAZ family genes, like Vasa homologs, have been used to mark germ cells across multiple species. A role for DAZ family genes in the progression through meiotic prophase I appears to be conserved from worms to mice, although in vertebrates (in particular zebrafish and frogs) there appears to be a further role in PGC development. That DAZ family genes are translational regulators has been demonstrated directly only in Xenopus (Xdazl). A link between a putative target of a DAZ family gene and a mutant phenotype has been demonstrated only in Drosophila (boule). A better understanding of the function of the DAZ family genes awaits a clearer understanding of the role of vertebrate Dazl in germ cell development and also of DAZ family protein biochemistry that can be linked to the function ascribed to them by genetic studies.

v) Conclusion

The extent of conservation of these germ cell gene families, in terms of sequence and, in some cases, even of function, is remarkable in light of the distance from worms to mammals. This observed conservation has led to a push to tap into the relatively large literature on Drosophila, C. elegans and Xenopus homologs to provide starting points in the study of the function of mammalian homologs. A list of the genes examined and their
known expression pattern and functions has been compiled for ease of comparison between various genes over various species (Table 1).

Just as meiosis genes conserved from yeast to humans inform us about what the ancient and fundamental processes of meiosis are, so too do the conserved early germ line development genes offer us some insights into features that distinguish early germ cell development from other developmental processes. Thus far, the theme that resonates most clearly through the studies of these genes is the need to preserve the undifferentiated state of the future germ line, to prevent their differentiating down somatic pathways.

Germ cells appear to have multiple mechanisms in place to preserving their undifferentiated state. In some cases, this is achieved by transcriptional quiescence: Shutting down RNA Pol II activity or having specific chromatin structures that maintain global transcriptional silencing are two methods employed to achieve this. In some cases, translational silencing of genes by *nanos* and *pumilio* homologs is a means to repress expression of somatic genes. In the case of mice, germ cells maintain pluripotency-related genes like *Oct4* many days beyond any other cell type in the embryo (Pesce et al., 1998), although whether this is a cause or effect of maintaining an undifferentiated state is unclear.
Table 1: List of some gene families involved in early germ cell development, including family members in various species, their known expression patterns and functions— in particular those pertaining to germ cell development.
Table 1:

<table>
<thead>
<tr>
<th>Gene family</th>
<th>Gene name</th>
<th>Species</th>
<th>Expression pattern</th>
<th>Known functions (with emphasis on functions in the germ line)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nos-1</td>
<td>Caenorhabditis elegans</td>
<td>-</td>
<td>Required for the efficient entry of germ cells into somatic gonad.</td>
<td>[1]</td>
</tr>
<tr>
<td></td>
<td>nos-2</td>
<td>Caenorhabditis elegans</td>
<td>-</td>
<td>Required for larval germ cell viability and prevents ectopic divisions of germ cells.</td>
<td>[1]</td>
</tr>
<tr>
<td></td>
<td>nos-3</td>
<td>Caenorhabditis elegans</td>
<td>-</td>
<td>Functions in the control of the mitosis-meiosis switch as well as the sperm-oocyte switch (although this later function is partially redundant with nos-1 and nos-2).</td>
<td>[2]</td>
</tr>
<tr>
<td>Nanos</td>
<td>nos</td>
<td>Drosophila melanogaster</td>
<td>Posterior of early embryo and subsequently localizes to pole plasm</td>
<td>Maternal nos, along with pum, is required for posterior patterning in the embryo, maintenance of pole cell survival and various pole cell characteristics including transcriptional and mitotic quiescence; zygotic nos is required for germ line stem cell self-renewal in the ovary.</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>Xcat2</td>
<td>Xenopus laevis</td>
<td>mRNA localizes to germ plasm in early embryo</td>
<td>Interacts with Pumilio to regulate CyclinB1 translation</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>Nanos-1</td>
<td>Mus musculus</td>
<td>Primarily in the CNS with some adult ovarian and testis expression</td>
<td>Suspected to have redundant functions as the knockout mice are normal.</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>Nanos-2</td>
<td>Mus musculus</td>
<td>Only expressed in XY embryonic germ cells between E13.5 and E16.5</td>
<td>Embryonic XY germ cell survival and localization within cords.</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>Nanos-3</td>
<td>Mus musculus</td>
<td>Only expressed in XX and XY embryonic germ cells prior to E15.5</td>
<td>Embryonic XX and XY germ cell survival prior to E12.5</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>NOS1</td>
<td>Homo sapiens</td>
<td>Highly expressed in spermatogonia and early spermatocytes</td>
<td>-</td>
<td>[7]</td>
</tr>
<tr>
<td>PUF</td>
<td><strong>Puf6p</strong></td>
<td><strong>Saccharomyces cerevisiae</strong></td>
<td>Puf6p protein is a component of the <strong>ASH1 mRNP</strong></td>
<td>Represses <strong>ASH1</strong> (a determinant for mating-type switching) translation</td>
<td>[8]</td>
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<tr>
<td><strong>DjPum</strong></td>
<td><strong>Dugesia japonica</strong> (Planarian)</td>
<td>Preferentially expressed in neoblasts (adult, totipotent stem cells)</td>
<td>Required for neoblast maintenance</td>
<td></td>
<td>[9]</td>
</tr>
<tr>
<td><strong>fbf-1/2</strong></td>
<td><strong>Caenorhabditis elegans</strong></td>
<td>Fbf-1 protein is expressed primarily in mitotic germ cells</td>
<td>Required to maintain self-renewing potential of germ line stem cells, regulate the size of the mitotic region in the gonad, and promote the sperm-to-oocyte transition</td>
<td></td>
<td>[10]</td>
</tr>
<tr>
<td><strong>Puf-8</strong></td>
<td><strong>Caenorhabditis elegans</strong></td>
<td>Primarily in the germ line</td>
<td>Required in spermatocytes to maintain meiosis and prevent a return to mitosis</td>
<td></td>
<td>[11]</td>
</tr>
<tr>
<td><strong>pum</strong></td>
<td><strong>Drosophila melanogaster</strong></td>
<td>Widely expressed, including in pole plasm</td>
<td>Maternal <strong>pum</strong>, along with <strong>nos</strong>, is required for posterior patterning in the embryo, maintenance of pole cell survival and various pole cell characteristics including transcriptional and mitotic quiescence; zygotic <strong>pum</strong> is required for asymmetric division of ovarian germ line stem cells.</td>
<td></td>
<td>[12]</td>
</tr>
<tr>
<td><strong>Xpum</strong></td>
<td><strong>Xenopus laevis</strong></td>
<td>-</td>
<td>Biochemically defined role in translational repression of <strong>CyclinB1</strong> in oocytes possibly via interaction with CPEB and Xcat proteins.</td>
<td></td>
<td>[13]</td>
</tr>
<tr>
<td><strong>pum-2</strong></td>
<td><strong>Mus musculus</strong></td>
<td>Widely expressed, but highly expressed in embryonic germ cells and spermatogonia, spermatocytes and oocytes of adult gonads.</td>
<td>-</td>
<td></td>
<td>[14]</td>
</tr>
<tr>
<td>PUM-2</td>
<td><strong>Homo sapiens</strong></td>
<td>Widely expressed, but highly expressed in germ cells and ES cells.</td>
<td>PUM2 protein interacts in vitro with NOS-1, DAZ and DAZL proteins, as well as the Nanos Response Element RNA sequence.</td>
<td></td>
<td>[15]</td>
</tr>
<tr>
<td>Vasa</td>
<td>Drosophila melanogaster</td>
<td>Initially protein is found all over oocyte, eventually protein and mRNA localize to the pole plasm</td>
<td>Required for posterior segment patterning and pole plasm assembly, including localization and translation of <em>nanos</em></td>
<td>[16]</td>
<td></td>
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<tr>
<td>glh-1-4</td>
<td>Caenorhabditis elegans</td>
<td>-</td>
<td>Required for development of sperm and survival of oocytes in hermaphrodites</td>
<td>[13]</td>
<td></td>
</tr>
<tr>
<td>XVLG</td>
<td>Xenopus laevis</td>
<td>Localizes to pole plasm</td>
<td>Required for PGC differentiation at the tadpole stage, although PGC formation is normal.</td>
<td>[17]</td>
<td></td>
</tr>
<tr>
<td>vas</td>
<td>Danio rerio</td>
<td>mRNA localizes to germ plasm, although maternal protein is more widely expressed in the early embryo. Subsequently both maternal and zygotic vas localize to the primordial germ cells.</td>
<td>-</td>
<td>[18]</td>
<td></td>
</tr>
<tr>
<td>Cvh</td>
<td>Gallus gallus</td>
<td>Protein is found in germ line cells from PGCs to spermatid and oocytes; within oocytes protein is found in structures that resemble germ plasm.</td>
<td>-</td>
<td>[19]</td>
<td></td>
</tr>
<tr>
<td>Mvh</td>
<td>Mus musculus</td>
<td>mRNA and protein are expressed in primordial germ cells upon interaction with the somatic gonad.</td>
<td>Role in XY embryonic germ cell survival and required for progression through prophase 1 of meiosis in males.</td>
<td>[20]</td>
<td></td>
</tr>
<tr>
<td>DAZ</td>
<td>daz-1</td>
<td>Caenorhabditis elegans</td>
<td>Protein is expressed in both male and hermaphrodite mitotic and early meiotic germ cells</td>
<td>Required in hermaphrodites for oocyte progression through pachytene of meiosis 1, and for premeiotic nuclear organization events in oogenesis.</td>
<td>[21]</td>
</tr>
<tr>
<td>DAZ</td>
<td>boule</td>
<td>Drosophila melanogaster</td>
<td>Required for progression through G2-M of meiosis1 in males via translational upregulation of <em>twine</em> (drosophila, meiosis-specific <em>Cdc25</em>), also required for events in spermiogenesis.</td>
<td></td>
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<td></td>
<td><em>Xdazl</em></td>
<td><em>Xenopus laevis</em></td>
<td>mRNA localizes to germ plasm, protein is observed from the blastulae stage and persists in PGCs until the early tail bud stage. Required for PGC differentiation, migration and survival in tadpoles, but not for germ plasm aggregation or PGC formation.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td><em>zDazl</em></td>
<td><em>Danio rerio</em></td>
<td>mRNA localizes to germ plasm, protein appears in primordial germ cells at genital ridge and is present in all germ cells after that point except for mature spermatozoa. In vitro, ZDAZL protein activates translation of <em>twine</em> and <em>zDazl</em> mRNAs dependent on the (GUUC)n motif on their 3’ UTRs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Boule</em></td>
<td><em>Mus musculus</em></td>
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<td><em>Dazl</em></td>
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<td>mRNA and protein expressed in primordial germ cells, spermatogonia and spermatocytes. Required for male and female germ cells to progress through meiotic prophase.</td>
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<td><em>BOULE</em></td>
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<td><em>DAZL</em></td>
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<td>PGCs, oocytes and adult male germ cells express <em>DAZL</em> mRNA, DAZL protein is expressed from spermatogonia through spermatid. -</td>
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<td>Homo sapiens</td>
<td>mRNA and protein are found in spermatagonia and spermatocytes, protein has been observed in PGCs, spermatogonia and spermatocytes but not later stage germ cells.</td>
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* Human DAZ is Y-linked, and hence its expression and functions are expected to be male-specific.

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A deeper understanding of the functions of these early germ cell differentiation genes will lead us to more themes by which we can define early germ line cells and understand some of the processes that enable this lineage to be uniquely immortal.

C) Commitment to Gametogenic, Sexually Dimorphic Fate

At some point in germ cell development all of the complex regulatory mechanisms in place to preserve the germ line cells in an undifferentiated state are reversed, and the germ cells switch to the implementation of processes that lead to terminal differentiation and gamete formation. These processes include, but are not limited to, meiosis, loss of pluripotency and sexual differentiation. In this section I review what is known about the initiation of these terminal differentiation processes in murine embryonic germ cells.

Mouse embryonic germ cells can be broadly divided into three types: migrating PGCs, gonocytes, and sexually dimorphic germ cells. The temporal relationship between these cell types and a pictorial representation of their sectioned, histological morphology as well as some markers of their fate are schematized in Fig. 1. Briefly, from embryonic day 7.25 (E7.25), when we can first visualize murine germ cells as AP-positive cells, PGCs migrate from their position at the base of the allantois toward the genital ridges, which eventually form the gonads. They complete their migration into the somatic gonad by E10.5-E11.5 and become gonocytes. Here they become less motile, express markers like N-Cadherin, Germ Cell Nuclear Antigen (GCNA) and Mvh (Enders and May, 1994; Pesce et al., 1998; Toyooka et al., 2000).
Up to E13.5, XX and XY germ cells are morphologically indistinguishable. However, by E14.5, XX and XY germ cells are clearly sexually dimorphic (Fig. 2). Furthermore, it is known that embryonic germ (EG) cell colonies (pluripotent cell colonies made from the culture of embryonic germ cells) can be made only from germ cells before E13.5 (Labosky et al., 1994b). To study how sexually indifferent, pluripotent embryonic germ cells transition into sexually dimorphic germ cells, destined for a gamete-producing fate, I was led to more closely examine E10.5 to E14.5 murine germ cells.

I define “sexually dimorphic, differentiated germ cells” as those germ cells that cannot be induced (even by cell culture) to give rise to any other cells than gametes of a particular gender. Given that, sexually dimorphic, differentiated germ cells appear in mice sometime between E10.5 and E14.5- when gonocytes transition to become sexually dimorphic germ cells. To better understand this transition, I review here the developmental changes that take place in germ cells between E10.5 and E14.5, including changes in germ cell morphology, cell cycle, gene expression and pluripotency/differentiation state, with particular attention to sexually dimorphic changes. I will also discuss some of the dramatic changes occurring in the somatic gonad at this time point, and what is understood about germ cell and somatic cell developmental interactions.
Fig. 2: Diagram outlining major processes of murine embryonic germ cell development with particular emphasis on the gonocyte-to-sexually-dimorphic-germ-cell transition. (A) Diagram representing the “Bucky ball” morphology common to XX and XY germ cells at E12.5-E13.5. (B) Diagram representing the morphology of XY germ cell in proliferation arrest, with diffused chromatin and prominent nucleolus. (C) Diagram representing the morphology of XX germ cell in meiotic prophase, with condensed chromatin in string-like structures centered within the nucleus. (D) Three-dimensional representation of a C-60 atom, otherwise known as a “Bucky Ball”, which, from the histological pictures, might be what XX and XY germ cells’ chromatin look like at E12.5-E13.5. (E) The mRNA/protein expression pattern of various genes in embryonic germ cells from E7.25 (when germ cells can first be observed) to D0.5 (birth). Black represents “not expressed” while blue represents “highly expressed”. Genes are clustered according to the germ cell developmental stage they represent. Dazl, Mvh and GCNA are gonocyte markers (they appear between when germ cells enter the somatic gonad and prior to sexual dimorphism). Stra8, Dmc1, SCP3 and γH2AX are markers of meiosis. Oct4 and Dppa3/Stella are makers of pluripotency.
Migrating Germ Cells  Gonocytes  Sexually Dimorphic Germ Cells

E7.25 ... E10.5  E12.5  E14.5  E16.5 ... D0.5

D

C_50 structure

B

Proliferation arrest

Nucleolus

C

Meiosis

Nucleus  Chromatin

E Expression of genes in XX germ cells

<table>
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<tr>
<th>Gene</th>
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i) Morphology

Morphologically, germ cells are difficult to distinguish from surrounding somatic cells up to, and including, E12.5. At E12.5-E13.5 a cell type common to both XX and XY germ cells, but distinct from the surrounding somatic cells appears. This cell is characterized by chromatin condensed in spots at the periphery of the nucleus, leaving the center of the nuclei negative to hematoxylin staining (see Fig. 2A). Henceforth, this cell type will be referred to as a “bucky ball” because of their resemblance to Carbon-60 (C\textsubscript{60}) molecules (bucky balls). From the appearance of the cell type in histological sections, I imagine that the hematoxylin-dark spots arranged around the nuclear periphery, with some threads of chromatin leading from it to other spots would look much as the molecular structure of the C\textsubscript{60} does (Fig. 2D) (David et al., 1991). It has been previously reported that these nuclei contain about 40 discrete spots of chromatin, which is consistent with the idea that each spot might correspond to a single chromosome.

Premeiotic condensation into chromosome territories has been observed in other organisms (ranging from \textit{Drosophila} to yeast and mice) and is postulated to exist in most species (Scherthan et al., 1994; Scherthan et al., 1996; Vazquez et al., 2002). In \textit{Drosophila} these spots are referred to as chromosome domains. They correspond to individual chromosomes and are thought to be grouped as such so that correct pairing between homologs can be established as the cells progress toward meiosis and potential mispairing with other chromosomes is minimized (Vazquez et al., 2002).

Beyond E13.5, XX and XY germ cells acquire dimorphic morphologies. The chromatin in XX germ cells become condensed into thread-like structures as they enter meiotic prophase (forming so-called meiotic figures, Fig 2B), while the chromatin in XY
germ cells will become diffused and a prominent nucleolus can be observed (see Fig. 2C). Bucky balls are the last common morphological cell type between XX and XY germ cells (Adams and McLaren, 2002).

ii) Meiosis and Proliferation arrest

As XX and XY germ cells diverge morphologically, they also take on different cell cycle properties. Previous to E12.5, gonocytes undergo several rounds of mitotic division after reaching the genital ridge. However, from E13.5 onward, both XX and XY germ cells gradually cease proliferating. XX germ cells enter meiosis and XY germ cells enter a state of proliferation arrest (McLaren, 1984; Peters, 1970). As the last cell type morphologically common to both XX and XY germ cells, bucky balls are thought to be at the point when germ cells decide whether to take on a meiotic female fate, or a proliferation-arrested male fate (Adams and McLaren, 2002).

Although quite a few mouse mutants defective in meiotic prophase are known (see Fig. 3 for a summary of some genes known to be required for progression through meiosis), not much is known about the molecular machinery that controls the entry into meiosis or proliferation arrest in females and males respectively. However two mutants are of interest in reference to these decisions, namely the Stimulated by Retinoic Acid Gene-8 (Stra8) and p27Kip1 mutant mice.

Stra8 was first identified as a transcript expressed by Embryonic Carcinoma (EC) cells in response to exposure to retinoic acid (Oulad-Abdelghani et al., 1996). It was first documented to be a male-germ-cell-specific gene based on its adult expression in mice,
Fig. 3: Diagrammatic representation of the mitotic and meiotic cell cycles of male and female germ cells, showing the stages at which the deletion of various genes disrupts the development of mutant germ cells.
Spermatogenesis

Oogenesis

Adapted from Fig. 1 of "Insights into regulation of the mammalian cell cycle from studies on spermatogenesis using genetic approaches in animal models" Wolgemuth, 2003 and Table 1 of "Regulation of Mitotic and Meiotic Cell Cycles in the Male Germ Line" Wolgemuth et al., 2002

Figure references:
Barlow et al., 1998
Baudat et al., 2000
Beumer et al., 1999
Dickens et al., 2002
Dix et al., 1997
Edelmann et al., 1996
Guardavaccaro et al., 2003
Kneitz et al., 2000
Liu et al., 1998
Romanienko and Camerini-Otero, 2000
Toscani et al., 1997
Wolgemuth et al., 2002
Wolgemuth, 2003
Woods et al., 1999
Xu et al., 1996
Yoshida et al., 1998
Yuan et al., 2000
but found to be female-germ-cell-specific in embryonic gonads. In fact, its expression correlates in both male and female germ cells with entry into the first meiotic prophase. In XX gonads, it is up-regulated and then down-regulated in an anterior-to-posterior (AP) wave starting from around E12.5 and ending around E16.5 (Menke et al., 2003). As mentioned previously, Stra8 is the earliest known female-germ-cell-specific gene, and its expression precedes the meiotic prophase genes Dmc1, γH2AX and SCP3 in embryonic XX gonads. Dmc1 is the meiosis-specific homolog of bacterial RecA (Sato et al., 1995). Bacterial RecA catalyzes the ssDNA invasion of homologous dsDNA, branch migration as well as the formation of Holiday junctions (Kowalczykowski, 1991). Dmc1 is known to localize to recombination nodules on meiotic chromosomes in prophase of meiosis I. Dmc1 knockout germ cells do not progress beyond zygotene and display defects in chromosome synapsis as well as formation of mature synaptonemal complexes (Pittman et al., 1998; Yoshida et al., 1998b). γH2AX is the phosphorylated form of the histone H2AX protein, conserved from yeast to mammals, that marks double strand breaks in DNA (Redon et al., 2002). Synaptonemal complexes are highly conserved structures found in all sexually reproducing eukaryotes that hold homologous chromosomes in close proximity and are required for recombination to take place. Synaptonemal complex protein 3 (Scp3) is a component of the axial element of the synaptonemal complex, and is one of the first to be loaded onto chromatin during murine meiotic prophase (Yuan et al., 2000).

Stra8 expression is required for the expression of all three of these meiotic prophase genes, and for the formation of morphologically meiotic germ cells (Andrew Baltus, personal communication). Stra8 knockout XX germ cells do not progress beyond
the bucky ball stage morphologically and do not express Dmc1, γH2AX or SCP3, nor do they properly carry out pre-meiotic S-phase (Andrew Baltus, personal communication). From this, it would appear that Stra8 is required for XX germ cells to initiate pre-meiotic S-phase. Stra8 knockout male germ cells also appear to have early meiotic defects although it is not yet clear how closely this phenotype mimics the phenotype of the Stra8 knockout female germ cells. (Andrew Baltus, personal communication). Amongst the many mutants that affect meiosis, Stra8 mutants have the earliest observed phenotype, particularly in females.

Not much is known about what regulates the proliferation arrest undergone by XY germ cells embryonically, but p27kip1 mutant mice display an interesting phenotype with regard to this proliferation arrest. p27kip1 is a cyclin dependent kinase inhibitor. It acts to oppose mitotic proliferation and is found to be highly expressed in many types of differentiated, or proliferation arrested cells. Indeed, p27kip mutant mice are larger than wildtype mice because so many organs in the mutant mice have more cells than wildtype mice. Further, mice lacking even one copy of p27kip1 are tumor-susceptible (Fero et al., 1998). P27KIP1 protein is expressed in XY germ cells starting around E16.5, when proliferation arrest is complete. Even though p27kip1 knockout XY germ cells can undergo proliferation arrest, the p27kip1 knockout mice have an excess of spermatogonia at birth, and it has been speculated that the embryonic proliferation arrest is either delayed or incomplete due to the lack of p27kip. This implies that, in late XY embryonic germ cells, p27kip plays a role in initiation or maintenance of proliferation arrest (Beumer et al., 1999). It is not known if p27kip1 is expressed in embryonic XX germ cells.
The timing of meiosis is the key difference between XX and XY germ cells I emphasize here, but interestingly, mutations that disrupt meiosis also impact XX and XY germ cells differentially (for a summary of some meiotic genes whose disruption gives different phenotypes in male and female germ cells, see Fig 3). It is probable that the process and regulation of meiosis is itself sexually dimorphic. One obvious reason why this may be true is that in males meiosis proceeds through to completion once initiated, but in females meiosis arrests at two points, diplotene of meiosis 1, in which state germ cells stay arrested for many months, and then metaphase of meiosis 2 which is completed only upon fertilization (Wolgemuth et al., 2002).

Another interesting point is that murine mutants that have phenotypes in meiotic prophase do not all have germ cells which arrest or apoptose at the same stage (Fig. 3)-implying that if checkpoints are present in mammalian meiosis, there are multiple checkpoints in prophase 1 (Wolgemuth et al., 2002).

The G2-M transition is an early meiotic transition whose regulation is understood to some degree, at least in the male. This transition is controlled by the M-phase promoting factor (MPF), which is made up of Cyclin-dependent kinase1 (CDK1) and Cyclin B (Chapman and Wolgemuth, 1994). In G2, CDK1 is present and found in a complex with Cyclin B but CDK1 is tri-phosphorylated and hence inactive (two of these phosphorylations- T16 and Y15- are inhibitory). At the onset of M phase, CDC25 phosphatase removes the inhibitory phosphates, activating the CDK1-Cyclin B complex (reviewed in Nurse, 1990). CDC25 itself is activated by phosphorylation by this activated complex, leading to a positive feedback loop (Hoffmann et al., 1993) (For review, see Fig 4 and Wolgemuth et al., 2002)
Fig. 4: Diagrammatic representation of the regulation involved in activation of the MPF (CDK1-CyclinB complex) at the G2-M transition in murine male meiosis.
Figure from Fig. 3 of Regulation of the Mitotic and Meiotic Cell Cycles in the Male Germ Line Wolgemuth et al., 2002
ii) Gene expression

Although morphologically it is difficult to tell XX and XY germ cells apart at E13.5, there are reports of expression differences between XX and XY germ cells as early as E11.5. The first difference in a germ-cell-specific gene, however, occurs at E12.5. *Stra8*, appear starting from E12.5 in XX but not XY germ cells (Menke et al., 2003).

As mentioned above, within a few days of Stra8’s expression, genes known to be involved in meiotic progression are expressed in embryonic XX, but not XY, germ cells. About a day after the appearance of *Stra8* in an anterior-to-posterior wave sweeping across the XX gonad, two genes associated with pluripotency, *Oct4* and *Dppa3/Stella* begin to be down-regulated in a retreating anterior-to-posterior wave in XX, but not XY, gonads (Bowles et al., 2003b; Menke et al., 2003). *Oct4* is a member of the POU family of transcriptional regulators. It is expressed only in pluripotent cells, zygotes, the inner cell mass (ICM), early germ line cells and ES cells. The pluripotency of ES cells has been shown to be sensitive to the amount of *Oct4* expressed, and it has been shown to regulate other stem cell-specific genes, hence it has been used as a defining marker of pluripotent cells (Nishimoto et al., 1999; Niwa et al., 2000). *Dppa3/Stella* (also known as *Pgc7*) was identified in various searches for pluripotency-related genes and is expressed in pre-implantation embryos, ES cells, premeiotic germ cells, but also ovaries from newborn mice. *Dppa3/Stella* appears in germ cells right around the time we can identify them (E7.25) and persists until around E13.5 when, like *Oct4*, it is down-regulated in an anterior-to-posterior wave in germ cells in XX gonads (Bowles et al., 2003b; Saitou et al., 2002). A third pluripotency associated gene which is downregulated at this time point is
Nanog. Several aspects of Nanog expression are intriguing. NANOG protein is downregulated in both XX and XY germ cells. In XX germ cells, NANOG is reduced by E13.5, while in XY germ cells the protein persists for a day or so more. Its downregulation is thus earlier than Oct4 or Dppa3/Stella in both XX and XY germ cells, and its expression appears to coincide with mitotically active embryonic germ cells since (unlike Oct4 and Dppa3/Stella), it is not upregulated in maturing oocytes postnatally (Yamaguchi et. al., 2005). Potentially, these characteristics make Nanog a better marker for the commitment to gametogenic fate experienced by both XX and XY germ cells around E12.5-E14.5 than either Oct4 or Dppa3/Stella.

iii) Germ Cell and Somatic Cell Sex

Germ cell sexual fate is thought to be derived from the somatic environment rather than by a germ cell’s own genetic composition. This commitment is thought to take place at E12.5 and E13.5 for XX and XY germ cells respectively, about the time that bucky balls appear.

The evidence that somatic environment is the sex determinant for germ cells comes from various transgene, transplantation and chimera experiments. The presence of Sry, a gene which codes for a transcription factor found on eutherian Y chromosomes, as a transgene in XX mice is sufficient for complete somatic sex-reversal (Koopman et al., 1991). Sry is therefore considered the male sex-determining gene. Sry is expressed in the Sertoli-cell lineage and only from about E10.5- E12.5. It has also been shown in XX-XY chimeric gonads that the Sertoli cells were the cells that were biased to be XY rather
than XX (Burgoyne et al., 1988). Together, these data are a powerful argument for the Sertoli cells being the primary cell type determinant of testis fate.

From transplantation experiments, it is known that the chromosomal sex of the surrounding somatic gonad determines a germ cell’s sexual characteristics. XX germ cells taken from gonads before E13.5 can adopt the diffused chromatin morphology associated with male, proliferation-arrested germ cells when transplanted into an XY gonadal environment. Conversely XY germ cells taken from gonads before E12.5 can adopt a meiotic morphology associated with female germ cells when transplanted into an XX gonad environment. However XX germ cells at E13.5 and XY germ cells at E12.5 can no longer respond to the somatic environment by changing their sexual fate. From this data it was concluded that events that commit an XX germ cell to a feminine fate happen prior to E13.5, while events that commit XY germ cells to a masculine fate happen prior to E12.5 (Adams and McLaren, 2002).

Even though in the testis it is clear that somatic sex determines germ cell sexual identity, oocytes are thought to play an active role in the organization of the fundamentally female somatic structure in ovaries, the follicle (Matzuk et al., 2002). When XY embryos are depleted of germ cells prior to the formation of a testis from the bipotential genital ridge, testis cords form and are present through adulthood. On the other hand, without germ cells XX gonads develop into streaks of connective tissue. Removing oocytes from follicles causes the follicles to degrade, and a germ-cell-specific transcription factor, FlGa, is required for follicle formation (McLaren, 1988; Soyal et al., 2000). These results have been taken to imply that meiotic XX germ cells actively organize the somatic ovary. At least one case of germ cells feminizing somatic cells is
known in the study of vertebrate sexual differentiation. Ablating PGCs in zebrafish invariably gives rise to male zebrafish. However, this effect is attributed to the fact that gonads are missing in individuals that lack PGCs, and hence lack certain sex hormones (Slanchev et al., 2005).

The question is how sertoli cells in an embryonic testis communicate their chromosomal sex to the other cell lineages (including germ cells) in an embryonic testis. A hypothesis was put forward recently after the observation was made that Prostaglandin D2 synthase mRNA is highly expressed in testis cords starting from E12.5, in both the germ cells and Sertoli cells, while it is not expressed in any cells within the female gonad at E12.5. Interestingly, the addition of endogenous Prostaglandin D2 masculinizes female gonads in culture, inducing cord-like structures and expression of anti-Mullerian hormone, a hormone produced by Sertoli cells that is required to oppose Mullerian duct formation in male sex differentiation. Most convincingly, the few germ cells that could be found in the masculinized regions of these ovo-testes had a male morphological appearance. This led to the postulate that the Prostaglandin D2 signal from Sertoli cells induces male differentiation in germ cells within XY gonads (Adams and McLaren, 2002). These results still leave open the question about what sorts of signals, if any, induce female differentiation in germ cells within an XX gonad.

Recent work suggests that signaling via retinoic acid receptors (RARs) is required to induce female-specific gene expression of Stra8 in embryonic germ cells. In whole gonad explant experiments it has been shown that culturing with RA induces Stra8 expression in XY germ cells, while culturing XX gonads with inhibitors of RARs suppress the expression of Stra8 and appearance of meiotic figures in XX germ cells.
Furthermore, culture with an inhibitor specific to CYP26B1, a cytochrome p450 that is known to specifically metabolize and reduce levels of active RA, induces E12.5 XY gonads to express Stra8 in a germ-cell-dependent manner. CYP26B1 is expressed in the somatic XY gonad, but not the XX gonad nor in germ cells. These data led to the hypothesis that it is the presence of CYB26B1 which blocks RA from reaching XY germ cells, preventing Stra8 expression and hence meiosis in XY germ cells. It remains to be seen if Stra8 expression, though required for meiosis, is sufficient to induce meiosis in XY germ cells, and whether the lack of Stra8, in turn, induces mitotic arrest. (Jana Koubova; personal communication). An intriguing question which arises from this work is why germ cells within the gonad are the only cell type in the embryo that respond to RA by expressing Stra8, since RA is known to be present in many other tissues.

In short, although there are some well-studied markers and determinants of testis development, the same cannot be said of ovarian development nor of germ cell sex determination and differentiation. The discovery of new markers, like Stra8, should give the field a starting point from which to explore these relatively neglected questions of sex differentiation.

iv) Exit from a pluripotent state

Although XX and XY germ cells carry out such different differentiation programs after E12.5, both exit from a pluripotent state at around this time point. It is easy to identify XX germ cells exiting from a pluripotent state. XX germ cells complete down-regulation of all pluripotency-associated genes studied at this time and enter the terminally differentiating process of meiosis. XY germ cells’ exit from a pluripotent state
is more subtle. Germ cells from both XX and XY embryonic mice at E12.5 or earlier can be cultured to give rise to EG (Embryonic Germ) cells. EG cells are pluripotent cells similar to ES cells in their ability to survive and divide to form colonies in culture and to be injected into the blast to give rise to all lineages in the mouse embryo. EG cells made from germ cells prior to E12.5 can give rise to all cell types except the extra-embryonic trophectoderm, while EG cells made from E12.5 germ cells have very limited differentiation potential. Germ cells after E12.5 lose their ability to give rise to EG cells (Labosky et al., 1994b; Stewart et al., 1994). Conversely, E14.5 XY germ cells, but not E12.5 XY germ cells, can initiate spermatogenesis when transplanted, without donor somatic cells, into adult testis (Ohta et al., 2004). These experiments imply that fundamental differentiation changes occurring between E11.5 and E13.5 lead to the loss of mitotic and pluripotent potential, and commitment to a sexually dimorphic gamete-producing fate in both XX and XY germ cells.

iv) Overview

In general, germ cell progenitors in metazoans are set aside early in embryonic development and maintained in an undifferentiated state until sometime after they come into contact with the somatic gonad. Beyond this point, a complex series of changes occurs in murine germ cells that lead to step-wise to loss of pluripotency, and commitment to a gametogenic, sexually dimorphic terminal differentiation program. This program culminates in meiosis and gamete formation. Although much is known about the process of meiosis itself, particularly molecular components involved in meiotic prophase that are highly conserved in eukaryotes, not as much attention has been
paid to the earlier processes of germ cell development. At first glance, these processes of
erg germ cell specification and pre-meiotic development appear less conserved across
metazoans. However, recent evidence of conserved gene families playing similar roles in
early germ cell development in multiple organisms has given the field starting points to
translate knowledge gathered from one model organism into experimental queries in
others.

In particular I was interested in understanding more fully, the role of murine Dazl
in germ cell development. DAZ family genes are so well conserved, and the phenotype
of deficiencies in vertebrates other than mice (Xenopus and zebrafish), seemed to
indicate a conserved role for Dazl in embryonic, pre-meiotic germ cell development. In
addition murine Dazl is known to be expressed in embryonic germ cells starting as early
as E11.5—a time point at which some critical processes of germ cell development take
place. In the following chapters I discuss the results that I obtained from examining the
Dazl-deficiency allele (Dazl<sup>TMHgw</sup>) backcrossed onto a C57BL/6 background.

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Chapter 2

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

Yanfeng Lin and David C. Page
Abstract

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

Keywords: Dazl, DAZ, embryonic, Dazl knockout, pluripotency, germ cell, C57BL/6
Introduction

Genes in the Deleted in Azoospermia (DAZ) family are expressed specifically in germ cells and play a critical role in fertility in metazoans, including flies, worms, fish, frogs, and mammals (Eberhart et al., 1996; Hashimoto et al., 2004; Houston and King, 2000; Karashima et al., 2000; Reijo et al., 1995; Ruggiu et al., 1997; Xu et al., 2001). DAZ was first discovered in studies of the human Y chromosome, in a region whose deletion results in spermatogenic failure (Reijo et al., 1995). Since its discovery, studies of mouse Dazl have been directed primarily to its role in postnatal spermatogenesis, while the embryonic functions of Dazl in XY germ cells have remained largely unexplored (Maratou et al., 2004; Ruggiu et al., 1997; Saunders et al., 2003; Schrans-Stassen et al., 2001; Slee et al., 1999; Vogel et al., 2002). Disruptions of other vertebrate Dazl genes, including those in Xenopus and zebrafish, suggest however that Dazl might be crucial to the development and survival of embryonic germ cells (Hashimoto et al., 2004; Houston and King, 2000; Maegawa et al., 1999).

In mice, primordial germ cells arrive at the genital ridge around E10.5, and XY germ cells enter a state of proliferation arrest between E13.5 and E16.5 (Nagano et al., 2000). The mouse Dazl gene is first expressed at E11.5 in post-migratory germ cells (Seligman and Page, 1998). However, studies on mixed strain background mice did not detect defects in XY or XX Dazl -/- germ cells at E15.5 (Ruggiu et al., 1997). More recently, the timing of postnatal defects and death in XY Dazl -/- germ cells has been shown to vary, although significant numbers of germ cells were observed weeks after birth (Schrans-Stassen et al., 2001; Saunders et al., 2003). It is not possible to gauge how much of the observed variability in postnatal germ cell numbers and development is an
inherent characteristic of Dazl deficiency and how much is due to differences in unknown genetic modifier loci that may differ among strains (Saunders et al., 2003). To establish a reproducible standard for examining Dazl’s functions, we backcrossed the published Dazl knockout allele (Dazl<sup>Tmi/Hge</sup>) onto the C57BL/6 background. Our experiments indicated that Dazl is required for embryonic development and survival of XY germ cells as early as E12.5-E13.5.
Results

Dazl mRNA expression begins at E11.5

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

Neonatal Dazl -/- C57BL/6 testes contain very few germ cells

All 60 histological sections from testes of two wildtype XY neonates (D0.5) contained multiple germ cells per testis cord, and the chromatin of these germ cells, as expected, was diffuse rather than condensed (Fig. 2A and B). (Germ cells were distinguished from somatic cells by their central positions within testis cords, and by their larger nuclei.) By contrast, of 60 histological sections from testes of two Dazl -/- neonates, only two contained identifiable germ cells, and then only in two cord cross sections (Fig. 2C). These germ cells appeared abnormal, with chromatin condensed at the edge of the nucleus (Fig. 2D). As a more sensitive means of detecting germ cells in neonatal testes, we then assayed for expression of Mouse Vasa Homologue (MVH), a germ-cell-specific cytoplasmic protein. All ten testes sections examined from three wildtype neonates displayed multiple MVH-positive germ cells in each testis cord, with 100-200 germ cells in each cross section (Fig. 2E). By contrast, of eighteen testes sections with equivalent cross sectional areas from six Dazl -/- neonates, eleven
Fig. 1. Whole-mount in situ hybridization of *Dazl* riboprobe to wildtype XY embryonic gonads.
Fig. 2. Sections of neonatal (D0.5) testes from XY *Dazl* +/+ (A and E) and *Dazl* -/- (C and F) mice stained with hematoxylin and eosin (A and C, 40x magnification) or with MVH antisera (E and F, 10x magnification). Inserts show high magnification images of a typical D0.5 XY *Dazl* +/+ germ cell (B), and a rare D0.5 XY *Dazl* -/- germ cell (D). Arrowheads indicate germ cells.
contained no MVH-positive cells, five had 1-5 MVH-positive cells and one had 9 MVH-positive cells (Fig. 2F and data not shown).

Loss of germ cells occurs prenatally

As we observed virtually no germ cells in XY Dazl -/- testes at birth (n=4), we next examined the germ cell content of embryonic XY gonads. At E13.5, XY wildtype and Dazl -/- gonads could not be distinguished by light microscopic examination of histological sections (Fig. 3A and B). At E14.5, in XY Dazl -/- gonads, dark germ cells with a condensed nucleus, typical of apoptotic cells, were observed (n=2) (Fig. 3D). These cells were rarely seen in the gonads of wildtype XY littermates (Fig. 3C). By E15.5, many germ cells in XY Dazl -/- gonads appeared apoptotic (n=2) (Fig. 3F). At E17.5, many testis cords in XY Dazl -/- gonads contained only somatic cells, and few cords contained germ cells that did not appear apoptotic (n=3).

In wildtype XY germ cells, the chromatin gradually assumes a more diffuse appearance as the cells develop from E14.5 through birth (Fig. 3C, E, G and Fig. 2B). By contrast, the chromatin of surviving XY Dazl -/- germ cells maintained the condensed appearance typical of wildtype germ cells at E12.5-E13.5 (Fig. 3B, D, F and Fig. 2D).

Dramatic loss of germ cell markers at E14.5-E15.5 in Dazl -/- XY gonads

To confirm the embryonic loss of germ cells, we assayed expression of several germ-cell-specific markers (Mvh, Dppa3/Stella, Oct4, GCNA and MVH protein) in XY
Fig. 3. Hematoxylin and eosin stained sections of embryonic gonads from XY $Dazl$ $+/+$ (A, C, E and G) and $Dazl$ $-/-$ (B, D, F and H) littermate mice at E13.5 (A and B), E14.5 (C and D), E15.5 (E and F), and E17.5 (G and H). Arrowheads indicate germ cells. Arrows indicate presumptive apoptotic germ cells.
gonads, both wildtype and Dazl -/- (Bowles et al., 2003a; Fujiwara et al., 1994; Ovitt, 1998; Saitou et al., 2002).

At E14.5, we observed, via in situ hybridization, a decrease in the levels of Mvh, Oct4 and Dppa3/Stella transcripts in XY Dazl -/- gonads when compared to littermate controls (Fig. 4A-F). By E15.5, an even greater diminution of transcript levels was evident (data not shown). In addition, immunofluorescence experiments revealed that both MVH protein expression at E14.5 and GCNA expression at E15.5 were significantly reduced in XY Dazl -/- gonads when compared to wildtype controls (Fig. 4G-J).

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

The presence of apparently apoptotic germ cells led us to assay XY E15.5 gonads for apoptotic nuclei by fluorescent TUNEL analysis (Fig. 5A and B), using DAPI staining to identify testis cords and germ cell nuclei (Fig. 5C and D). We found that the number of TUNEL-positive cells was significantly increased in XY Dazl -/- embryonic gonads compared to littermate controls (Fig. 5A and B), despite the fact that the number of germ cell nuclei was decreased in XY Dazl -/- gonads (Fig. 5C and D). Further, most TUNEL-positive nuclei were found within testis cords (Fig. 5F). Thus, the TUNEL analysis confirms that apoptosis accounts for the dramatic loss of germ cells observed in XY Dazl -/- embryos.
Fig. 4. Staining for germ cell markers in XY Dazl +/- and Dazl -/- embryonic gonads. A-F, Whole-mount in situ hybridization of Mvh (A and B), Oct4 (C and D) or Dppa3/Stella (E and F) riboprobes to littermate E14.5 XY Dazl +/- (A, C and E) and Dazl -/- (B, D and F) gonads. G,H, Fluorescent immunohistochemistry reveals MVH-positive germ cells (red) on sections of E14.5 XY Dazl +/- (G) and Dazl -/- (H) gonads (10x magnification).  I,J, Fluorescent immunohistochemistry reveals GCNA-positive germ cells (green), and phosphohistone H3-positive (PH3) dividing cells (red) on sections of E15.5 XY Dazl +/- (I) and Dazl -/- (J) gonads (60x magnification).
Fig. 5. TUNEL and DAPI staining in E15.5 XY Dazl +/− (A, C and E) and Dazl −/− (B, D and F) gonad sections. TUNEL-positive cells visualized in green (A and B) and DAPI-positive nuclei in blue (C and D). Merged images in (E) and (F). Orange spots arise from non-specific fluorescence (A, B, E and F). Dotted white lines delineate some of the testis cords (C-F).
Discussion

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

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

In the present study, we observed that, at E15.5, most germ cells in XY Dazl -/- gonads appeared apoptotic, and the number of TUNEL-positive cells was elevated in Dazl -/- compared to Dazl +/- testes. In addition, several germ-cell-specific markers showed reduced expression in XY Dazl -/- gonads when compared to wildtype littermates at E14.5 and E15.5. In sum, on a C57BL6 strain background, germ cell loss in Dazl -/- gonads occurred as early as E14.5- E15.5.

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

Our findings stand in contrast to previous studies, all conducted in mixed genetic backgrounds, that suggest \textit{Dazl} is essential to XY germ cell development and survival only later in development, although reported time frames varied (Ruggiu et al., 1997; Schrans-Stassen et al., 2001; Saunders et al., 2003; Maratou et al., 2004). The earliest reported phenotype on a mixed genetic background is a drop in germ cell numbers at E19.5 (Ruggiu et al., 1997). A subsequent publication characterized the XY \textit{Dazl} -/- phenotype as a failure of postnatal spermatogonial differentiation, with no significant difference between germ cells of wildtype and \textit{Dazl} -/- testes until nine days after birth (Schrans-Stassen et al., 2001). A third publication found that the final defect in XY \textit{Dazl} -/- germ cell development is a block in prophase I of the first wave of meiosis, occurring one to two weeks after birth (Saunders et al., 2003). Most recently, a study comparing the RNA expression profiles of postnatal XY \textit{Dazl} -/- and wildtype testes concluded that leptotene-zygotene of meiosis is the most advanced stage attained by XY \textit{Dazl} -/- germ cells (Maratou et al., 2004).
These phenotypic studies of mixed background mice, buttressed by the fact that the highest levels of DAZL protein are observed in early spermatocytes, argue strongly that Dazl has important functions in postnatal XY germ cell development (Ruggiu et al., 1997). To explore these postnatal functions in greater detail, experiments must be carried out in a reproducible, pure-strain background (not yet identified) where XY Dazl -/- germ cells survive in the neonate; alternatively, a conditional Dazl allele must be made.

Our finding that Dazl is required in XY embryonic germ cells has implications for biochemical studies of the mouse DAZL protein. Previously, searches for mRNA binding partners of DAZL, a putative RNA-binding protein, have assumed that the adult testis is the relevant tissue in which the binding partners can be found (Jiao et al., 2002; Venables et al., 2001). However, these binding partners may be separable from to the ones Dazl binds to in mouse embryonic germ cells. We further suggest that microarray transcription profiling experiments in Dazl knockout mice be extended to embryonic XY gonads to explore Dazl’s prenatal function.

Variability in the timing of germ cell death in XY Dazl -/- (Dazl<sup>TM1/Hgu</sup>/Dazl<sup>TM1/Hgu</sup>) mixed background mice, and the earlier phenotype seen in our present study in mice of C57BL/6 background, indicate that strain background strongly affects the phenotype of Dazl -/- mice. Previous studies using mice of uncertain or mixed genetic background may need to be reinterpreted in light of this fact. For instance, in efforts to rescue the phenotype of XY Dazl -/- mice with human DAZ or DAZL transgenes, the human genes were inserted into ES cells from mice of different mixed backgrounds (Vogel et al., 2002). The degree of rescue observed with human DAZ transgenes was variable, and appeared to be independent of transgene copy number, insertion site and integrity of a
portion of the DAZ coding region (Vogel et al., 2002). It is possible that some portion of the effects attributed to the transgenes may actually represent the effects of strain-dependent modifier loci that were introduced along with the transgenes.

Sensitivity of an embryonic germ cell defect to strain background is not unique to Dazl knockout mice. Disruption of the Pinl gene resulted in no embryonic germ cell abnormalities until the mutation was bred into a C57BL/6 background, where both XY and XX Pinl-deficient embryos displayed poor germ cell proliferation (Atchison et al., 2003; Spears et al., 2003). In TrkB -/- neonatal ovaries, grossly abnormal morphology and reduced follicle numbers were observed in 50% of mixed background mice, but in 100% of C57BL/6 mice (Spears et al., 2003). Taken together, these results suggest that strain-dependence of mammalian embryonic germ cell phenotypes may be more common than previously appreciated. We believe that with the inbred C57BL/6 Dazl knockout mice, we now have the opportunity to study a newly identified requirement for Dazl at a time point where crucial, yet poorly understood, developmental changes occur in XY germ cells.
Materials and Methods

Backcrossing \( Dazl^{TM/Hm} \) onto C57BL/6 strain background

Mice carrying the allele \( Dazl^{TM/Hm} \) (Ruggiu et al., 1997) were kindly provided by Howard Cooke, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK. We crossed \( Dazl^{TM/Hm}/+ \) mice to C57BL/6 mice (Taconic Farms Inc., Germantown, NY). All resulting mice examined in the present study have Y chromosomes and mitochondria of C57BL/6 origin. All experiments were carried out on mice which were backcrossed into the C57BL/6 background between 7 and 11 generations, when 99.2% to 99.9% of the genome is expected to be of C57BL/6 origin.

Embryonic gonad collection and PCR-based genotyping

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

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

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

In situ hybridization

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

We thank Min Wu for the Dazl riboprobe; George Enders for the GCNA antibody; Toshiaki Noce for the MVH antisera; Mary Goodheart for animal husbandry; Michael Brown for processing and sectioning gonadal tissue samples; Steve Rozen, Alex Bortvin, Andrew Baltus and Steve Wasserman for advice and comments on the manuscript.
References


Chapter 3

Dazl enables germ cells of XX mouse embryos to Transition from pluripotency to meiosis

Yanfeng Lin, Jana C. Koubova and David C. Page

Author contributions:

Jana C. Koubova carried out the in situs that make up Fig. 6I and J.
Abstract

Germ cells can be defined as the cells that are fated to undergo the terminally differentiating process of meiosis. Mouse XX germ cells entering meiosis around 14.5 days postcoitum (E14.5) giving rise to meiotic figures, and down-regulate pluripotency markers. At this time, XY germ cells enter proliferation arrest, forming morphologically distinct nuclei. Mouse Dazl is a germ-cell-specific gene whose deletion causes germ cell loss. Here we report that the Dazl transcript is expressed in the XX gonad starting at around E11.5, similar to previous observations in XY gonads. XX Dazl -/- germ cells do not form meiotic figures, but instead maintain a morphology characteristic of wildtype E13.5 germ cells, similar to arrested XY Dazl -/- germ cells. These XX Dazl -/- germ cells also resemble XX germ cells deficient for Stra8, a gene required for the initiation of meiosis. Indeed we find that Dazl is required in embryonic XX germ cells for the expression of Stra8 and meiotic prophase markers SCP3, γH2AX and Dmc1. XX Dazl -/- germ cells also fail to down-regulate pluripotency markers Oct4 and Dppa3/Stella, distinct from XX Stra8 -/- germ cells that down-regulate Oct4 normally. We infer, therefore, that exit from a pluripotent state and entry into meiosis are separable processes, and that both require Dazl. We propose that Dazl functions at around E12.5 to allow XX germ cells to exit a pluripotent state, and acquire competence to undergo meiosis, a fundamental capability that defines germ cells. Further, we speculate that Dazl functions in both XX and XY germ cells to acquire differentiation competence, including the ability to undergo sex differentiation.
Introduction

Mouse Embryonic Germ Cell Differentiation between E11.5 and E14.5

In mice, embryonic germ cells can be divided broadly into three types: Migrating primordial germ cells (PGCs), gonocytes and sexually dimorphic germ cells. PGCs become gonocytes upon completing migration around E10.5, when they arrive at the bipotential gonad, become less motile and begin to express Germ Cell Nuclear Antigen (GCNA) and Mouse Vasa Homologue (Mvh) (Enders and May, 1994; Fujiwara et al., 1994; Toyooka et al., 2000). Gonocytes first exhibit sexually dimorphic morphology between E13.5 and E14.5 as XX germ cells enter meiosis and XY germ cells cease proliferation (McLaren, 2000) (Illustrated in Fig. 1).

Mouse germ cells undergo major differentiation events, including sexually dimorphic differentiation, between E11.5 and E14.5. Morphologically, XX and XY germ cells are difficult to distinguish before E13.5. Between E12.5 and E13.5, in both XX and XY gonads, a germ cell type with distinct nuclear morphology appears as chromatin condenses in a punctuated manner around the nuclear periphery (Fig. 1A) (McLaren and Southee, 1997). After E13.5, XX and XY germ cells have distinct morphologies. XX germ cells have condensed, thread-like chromatin centered in their relatively small nuclei (traits characterizing meiotic figures), while XY germ cells have prominent nucleoli and diffused chromatin spread out within larger nuclei (Fig. 1B, C).

XX and XY germ cells commit to their respective sexual fates at the time they share their last common morphology—punctuate chromatin at the nuclear periphery (see Fig 1A) (Adams and McLaren, 2002). Transplantation experiments suggest XX germ cells are committed to a morphologically female/meiotic fate by E13.5, while XY germ
cells are committed to a morphologically male fate by E12.5 (Adams and McLaren, 2002).

Though morphologically indistinguishable at E12.5, XX and XY germ cells have already begun to diverge in expression patterns. Transcription of the earliest known female-germ-cell-specific gene, \textit{Stra8}, has begun by E12.5 (Menke et al., 2003). In XX germ cells, meiotic prophase markers \textit{Dmc1} transcript, $\gamma$H2AX and SCP3 proteins appear soon after \textit{Stra8} expression (Dobson et al., 1994; Ikeya et al., 1996; Mahadevaiah et al., 2001; Yoshida et al., 1998a). \textit{Stra8} is required for the initiation of pre-meiotic S-phase and the expression or correct localization of these meiotic prophase markers (Andrew Baltus, personal communication). XX germ cells also begin to down-regulate \textit{Oct-4} and \textit{Dppa3/Stella} at around E13.5, while XY germ cells maintain expression of these pluripotency-associated genes (Bowles et al., 2003b; Menke et al., 2003; Pesce et al., 1998).

Although XY germ cells do not down-regulate pluripotency genes at E13.5 (Pesce et al., 1998), there is evidence that this is when XY germ cells commit to a spermatogenic fate. Germ cells from embryonic mice before E12.5 can be cultured to give totipotent Embryonic Germ cells (EG cells). However EG cells from E12.5 mice have limited differentiation potential, and EG cells have not been made from mice after E12.5 (Labosky et al., 1994b; Stewart et al., 1994). Conversely, E14.5 XY germ cells, but not E12.5 XY germ cells, can initiate spermatogenesis when transplanted without donor somatic cells into adult testis (Ohta et al., 2004). We infer from these experiments that fundamental differentiation changes occurring between E11.5 and E13.5 lead to loss of
pluripotency, and commitment to a sexually dimorphic, gametogenic fate in both XX and XY germ cells.

Expression of mouse Dazl and studies of the Dazl deficient mice

Given the host of differentiation events that occur between E11.5 and E14.5, it is noteworthy that the earliest embryonic Dazl transcript was detected by RT-PCR in germ cells at E11.5 (Seligman and Page, 1998). Indeed, in a recent microarray study, Dazl transcript was found to increase more than any other gene in mouse germ cells between E10.5 and E12.5 (Molyneaux et al., 2004).

Given Dazl’s early up-regulation, it is surprising that, until recently, the earliest defects observed in XX and XY Dazl -/- germ cells were seen at E19.5. E15.5 XX Dazl -/- germ cells were observed to have entered meiotic prophase, and both XX and XY germ cells showed normal numbers and morphology (Ruggiu et al., 1997). Most XX Dazl -/- germ cells were dead by E19.5, while there are substantial numbers of postnatal XY Dazl -/- germ cells, some surviving to be in meiotic prophase as late as 19 days postnatal (D19) (Maratou et al., 2004; Ruggiu et al., 1997; Saunders et al., 2003). It is clear, however, that genetic background heterogeneity has contributed to variability in survival and development of Dazl -/- germ cells in nearly all studies of the Dazl knockout (Saunders et al., 2003).

A recent study of Dazl -/- XY germ cell development in a C57BL/6 genetic background showed that Dazl -/- XY germ cells arrest at E13.5, within two days of Dazl expression. Dazl -/- XY germ cells do not develop beyond the morphology common to both XX and XY wildtype germ cells, and die prenatally (Lin and Page, in press). The onset of this phenotype is weeks before initiation of meiosis in normal males, and many
days earlier than the phenotype observed in XY $Dazl$-/- mice of mixed background (Ruggiu et al., 1997; Saunders et al., 2003).

In light of the timing of $Dazl$’s expression and the dramatic strain background influence on the XY $Dazl$-/- phenotype, we investigated the role of $Dazl$ in XX germ cell development between E12.5 and E14.5 on a C57BL/6 background. Specifically, we attempted to answer these questions: 1) Does $Dazl$ mRNA appear at the same time in wildtype XY and XX embryonic germ cells? 2) Do XX $Dazl$-/- germ cells, like XY $Dazl$-/- germ cells, demonstrate a morphological arrest, and if so when does the arrest occur in relative to early developmental events in wildtype XX germ cells? Do $Dazl$-/- XX germ cells become gonocytes? Can they enter meiosis or exit from the pluripotent state? 3) Are there similarities between the $Dazl$-/- XX and XY phenotype that may inform our understanding of early, XX-XY common functions for $Dazl$?
Results

*Dazl* mRNA expression in XX gonads begins around E11.5

Previously we examined XY C57BL/6 gonads and observed the earliest expression of *Dazl* mRNA between E11.5 and E12.5, as assayed by whole mount in situ hybridization (Lin and Page, in press). Here we used the same assay to determine the timing of *Dazl* mRNA expression in wildtype XX C57BL/6 embryonic gonads. At E11.5, a labeled riboprobe against *Dazl* mRNA produced no staining in most XX gonads, although a very faint staining could be seen in some (Fig. 2A). At E12.5, all XX gonads showed staining in the punctuated pattern characteristic of germ cell-specific transcripts (Fig. 2B). At this time point, *Dazl* expression in the XX gonads sometimes has an anterior-bias (Fig. 2B). By E13.5, *Dazl* transcript is highly expressed in germ cells in all parts of the XX gonads (Fig. 2C). Unlike meiosis-related genes, such as *Stra8* and *Dmc1* (Menke et al., 2003), *Dazl* transcript is not up-regulated in a clear anterior-posterior wave, nor does it disappear within a few days of its appearance, but rather it is present beyond E15.5 (Fig. 2D) (Ruggiu et al., 1997).

*Dazl*/- XX germ cells maintain E13.5 nuclear morphology, do not form meiotic figures, and grow abnormally large

We examined histological sections from XX gonads of littermate *Dazl*/- and wildtype mice at time points between E13.5 and birth (D0.5) to look for gross morphological differences.

Wildtype XX germ cells show characteristic nuclear morphological changes between E13.5 and birth as they enter meiotic prophase and arrest at diplotene of the first meiotic prophase (Fig. 3A, C, E, G, I). At E13.5, XX *Dazl*/- gonads could not be
**Fig. 1.** Diagrammatic representation of germ cell morphology at developmental stages E12.5 and E14.5 in mouse XX and XY embryos. Left, germ cell with punctuated chromatin at nuclear periphery, common to E12.5 and E13.5 XX and XY wildtype gonads; Top right, meiotic figures common to XX, but not XY, gonads starting at E14.; Bottom right, germ cell with diffused chromatin and prominent nucleolus, which appears in XY, but not XX, gonads starting at E14.5.
Fig. 2. Whole mount in situ hybridization with riboprobe against Dazl mRNA on E11.5, E12.5, E13.5 and E15.5 XX wildtype embryonic gonads. A faint staining could be observed in some of the E11.5 gonads, and by E12.5 all gonads.
Fig. 3. Histological sections of gonads from littermate XX $Dazl^{+/+}$ and $Dazl^{-/-}$ mice between E13.5 and D0.5 stained with hematoxylin and eosin. Arrows mark two representative germ cells in each section. Images were captured at 100x magnification.
distinguished by light microscope examination from XX wildtype littermate gonads (Fig. 3A, B).

The first clear morphological difference is observed at E14.5, when the chromatin of most XX wildtype germ cells has begun to condense into thin, thread-like structures, giving rise to meiotic figures (Fig. 3C). E14.5 XX Dazl -/- germ cells do not exhibit this morphology. Instead they retain the nuclear morphology of XX wildtype germ cells at E13.5, characterized by chromatin condensed in a punctuated manner around the periphery of the nucleus (Fig. 3D). XX Dazl -/- germ cells maintain this morphology beyond E14.5 although both their nuclei and cytoplasm grow abnormally large (Fig. 3F, H). Judging from their diameter, some Dazl -/- germ cells at D0.5 have volumes over an order of magnitude larger than littermate wildtype germ cells (Fig.3I, J). From their nuclear morphology, XX Dazl -/- germ cells arrest in the same cell type as both XY Dazl -/- germ cells and XX Stra8 -/- germ cells (Lin and Page, in press; Andrew Baltus, personal communication).

We did not observe a great excess of apoptotic figures in XX Dazl -/- gonads compared to wildtype littermate controls through birth. Although fewer germ cells are observed in D0.5 XX Dazl -/- gonads compared to wildtype littermates, a significant number of XX Dazl -/- germ cells survive until birth (Fig. 3F, H and J).

**XX Dazl -/- germ cells express MVH protein, but their morphologies and positions within the somatic gonad are abnormal**

Vasa family proteins are crucial for germ cell development in many organisms, and in mice, expression of *Mouse Vasa Homolog (Mvh)* marks the transition of germ cells to gonocytes (Toyooka et al., 2000). As MVH appears at about the same time as
Dazl transcript, we asked if MVH is expressed in Dazl -/- XX germ cells. We examined anti-MVH staining in sections from E15.5 and D0.5 XX Dazl -/- and littermate wildtype gonads. At E15.5, wildtype and Dazl -/- germ cells express MVH protein at similar levels (Fig. 4). By D0.5, there are fewer MVH-positive cells in XX Dazl -/- gonads compared to wildtype littermate gonads and expression in those MVH-positive cells in Dazl -/- gonads is lower than those in littermate XX wildtype gonads (Fig. 4).

Using MVH to identify germ cells, we were able to observe that D0.5 XX Dazl -/- gonads contain a defect in the positioning of germ cells within the neonatal gonad. D0.5 XX wildtype germ cells are positioned primarily in the cortex, close to the surface of the developing ovary (Fig. 4C). By contrast, XX Dazl -/- germ cells are distributed throughout the gonad (Fig. 4D).

**XX Dazl -/- germ cells do not express Stra8 or markers of meiotic prophase**

The XX Dazl -/- phenotype is similar to the XX Stra8 -/- phenotype in the morphology of the arrested germ cells. We therefore sought to understand the connection between Dazl and Stra8, first by assaying for Stra8 expression in XX Dazl -/- gonads.

XX wildtype germ cells express Stra8 transcript between E12.5 and E16.5 (Menke et al., 2003), and we found this to be true in the control E13.5 and E14.5 XX wildtype gonads (Fig. 5A, C). However, in situ hybridizations showed no evidence of Stra8 expression in littermate XX Dazl -/- gonads (Fig. 5B, D).

To corroborate our finding that XX Dazl -/- germ cells lack Stra8, we examined XX Dazl -/- germ cells for the expression of meiotic markers Dmc1, SCP3 and γH2AX which require Stra8. Dmc1 is the mouse homolog of RecA-like protein, and is involved
Fig. 4. Fluorescent immunohistochemical labeling of MVH (red) on Dazl +/+ (left two panels) and Dazl -/- (right two panels) XX gonad sections at E14.5 (top two panels) and D0.5 (bottom two panels). Images were captured at 40x (top two panels) or 10x (bottom two panels) magnification.
Fig. 5. Expression of Stra8 and meiotic prophase markers in XX Dazl -/- and wildtype littermate embryonic gonads. Whole mount in situ hybridization with riboprobes against Stra8 (A-D) or Dmc1 (E-H) mRNA on Dazl+/+ (A, C, E, G) and Dazl-/- (B, D, F, H) E13.5 (A, B), E14.5 (C, D, E, F) or E15.5 (G, H) XX embryonic gonads. Fluorescent immunohistochemical labeling against SCP3 (red, I, J) or γH2AX (red, K, L) on E15.5 Dazl +/- (I, K) and Dazl -/- (J, L) XX embryonic gonad sections. Images of gonad sections (I-L) were captured at 60x magnification.
in double-strand break repair and pairing of homologous chromosomes during meiosis (Sato et al., 1995; Pittman et al., 1998; Yoshida et al., 1998b). SCP3 is a part of the synaptonemal complex and γH2AX is a specifically phosphorylated form of histone H2A that marks double strand breaks in meiotic and apoptotic cells (Yuan et al., 2000; Redon et al., 2002). We found Dmc1 transcript, as expected, expressed in XX wildtype germ cells starting from E14.5 and peaking at E15.5 (Menke et al., 2003). We did not observe its transcript in littermate Dazl-/- gonads (Fig. 5F, H). Also as expected from the literature, we saw SCP3 and γH2AX highly expressed in the many germ cells in E15.5 XX wildtype gonads (Fig. 5I, K). Neither protein was observed in sections of XX Dazl-/- gonads, with the exception of rare, misshapen γH2AX-positive cells in the XX Dazl-/- gonads that we interpret to be apoptotic cells (Fig. 5J, L).

XX Dazl-/- germ cells erroneously maintain expression of markers of pluripotency

Coincident with the up-regulation of meiotic markers, XX wildtype germ cells also down-regulate markers of pluripotency between E13.5 and E15.5 (Bowles et al., 2003b; Menke et al., 2003). Mouse Oct4 and Dppa3/Stella are markers of pluripotency, and their down-regulation is correlated with differentiation. Indeed, Oct4 has a role in maintenance of stem cell characteristics in ES cells (Nichols et al., 1998; Niwa et al., 2000; Rosner et al., 1990). We observed that XX wildtype gonads down-regulate Oct4 and Dppa3/Stella by E15.5, consistent with earlier published finding (Fig. 6A, C, E, G). In contrast to this, germ cells throughout XX Dazl-/- gonads maintained high levels of Oct4 and Dppa3/Stella at E14.5 and E15.5 (Fig. 6B, D, F, H).
XX *Stra8* -/- germ cells down-regulate *Oct4* normally

* Stra8 is up-regulated about a day in advance of *Oct4* down-regulation. To test if the failure to down-regulate pluripotency genes could be, like the lack of meiotic markers, due to the lack of *Stra8* in XX *Dazl* -/- germ cells, we examined the expression of *Oct-4* in *Stra8* -/- gonads at E14.5 and E16.5. We found that *Stra8* -/- germ cells, in contrast to *Dazl* -/- germ cells, down-regulate *Oct-4* normally between E14.5 and E16.5 (Fig. 6I, J).
Fig. 6. Whole mount in situ hybridization with riboprobes against pluripotency markers Oct4 and Dppa3/Stella. In situ hybridization with riboprobes against Oct4 (A, B, E, F) or Dppa3/Stella (C, D, G, H) mRNA on E14.5 (A-D) or E15.5 (E-H) XX Dazl +/+ (A, C, E, G) and Dazl -/- (B, D, F, H) XX embryonic gonads. Whole mount in situ hybridization with riboprobes against Oct-4 mRNA on E14.5 (I) or E16.5 (J) XX Stra8 -/- embryonic gonads.
Discussion

We have shown here that in a C57BL/6 background, Dazl is first expressed between E11.5 and E12.5 in XX germ cells as it is in XY germ cells. We also show that XX Dazl -/- germ cells maintain a morphology that resembles that of arrested XY Dazl -/- and XX Stra8 -/- germ cells. We observed that XX Dazl -/- germ cells can express the gonocyte marker MVH and that a large fraction of them survive until birth, although at this point their morphology and position within the somatic gonad are abnormal. XX Dazl -/- germ cells do not express Stra8 or meiotic prophase markers Dmc1, SCP3 and γH2AX, nor do they down-regulate markers of pluripotency Oct4 and Dppa3/Stella. We found, however, that XX Stra8 -/- gonads down-regulate Oct4 in a normal fashion.

Dazl -/- germ cells become gonocytes

XX Dazl -/- germ cells are morphologically indistinguishable from XX wildtype germ cells through E13.5, which suggests that Dazl -/- germ cells can make a normal transition from migrating germ cell to gonocyte. The appearance of MVH protein, whose expression requires interaction between the germ cells and somatic gonad, corroborates this. We infer from this that Dazl is not required for germ cells to acquire at least some aspects of gonocyte identity.

Dazl is required for pluripotency exit and meiotic initiation, though these represent independent differentiation pathways

In XX germ cells, Dazl is required for expression of Stra8, which, in turn, is required for initiation of meiosis, implying that Dazl is required for meiotic initiation. Dazl is also required for XX germ cells to down-regulate pluripotency-associated genes Oct4 and Dppa3/Stella, which leads us to infer that Dazl is required for exit from a
pluripotent state. XX *Stra8* -/- germ cells down-regulate *Oct4* normally while XX *Dazl* -/- germ cells do not, indicating that *Stra8* is not required for events leading from *Dazl* expression to *Oct4* down-regulation. This suggests a model for the relationship between *Dazl*, meiotic initiation (as represented by *Stra8* expression) and exit from pluripotency (as represented by loss of *Oct4* transcript) (Fig. 7). The implication is that there are *Stra8*-independent (and hence meiosis-independent) pathways of differentiation in embryonic XX germ cells, and that exit from pluripotency is at least partly separable from entry into meiosis, even though both processes require *Dazl*.

**Dazl may be required for germ cell sex differentiation**

We interpret the presence of MVH and absence of meiotic markers in XX *Dazl* -/- germ cells to imply that differentiation arrest in *Dazl* -/- XX germ cells occurs after the germ cells have become gonocytes and prior to entry into meiosis, a process which distinguishes female from male embryonic germ cells. Traditionally, embryonic meiotic figures have been used to indicate germ cell female sexual fate, while the diffused-chromatin nuclear morphology is an indicator of germ cell male sexual fate (Chuma and Nakatsuji, 2001; McLaren and Southee, 1997). By these indicators, *Dazl* -/- XX and XY germ cells do not undergo sexual differentiation. In addition, XX *Dazl* -/- germ cells lack *Stra8*, the earliest known female-germ-cell-specific transcript (Menke et al., 2003). It is clear that *Dazl* is required for at least some sexually dimorphic differentiation in embryonic germ cells. In the absence of *Dazl*, sex-specific changes in morphology as well as in expression of meiosis genes and pluripotency-associated genes do not take place. However more molecular markers and a deeper understanding of sexual
differentiation in germ cells will be needed to explore the relationship between Dazl and
germ cell sex differentiation.

**Dazl’s regulation of entry into meiosis and down-regulation of Oct4 and**

**Dppa3/Stella is likely indirect**

It is likely that the relationships between Dazl and Oct4 or Stra8, as diagrammed in Fig.
7, are biochemically indirect. Mouse DAZL, like all other members of the DAZ family,
has an RNA recognition motif (RRM), and all available data suggests that DAZ family
proteins function as translational regulators (Maegawa et al., 2002; Maines and
Wasserman, 1999; Tsui et al., 2000). There is no evidence that DAZ family proteins can
affect transcription or stabilize mRNA transcript, hence we consider that the effects of
Dazl deficiency on Stra8, Oct4 and Dppa3/Stella transcripts likely to be mediated by at
least one intermediate biochemical step. It is possible that DAZL directly regulates SPC3
and γH2AX protein levels, but it is not necessary to postulate such a relationship as Stra8
deficiency alone results in the lack of meiotic prophase markers (Baltus et. al., 2005).
We conclude that Dazl functions at least one step upstream of both initiation of meiotic
prophase and down-regulation of pluripotency markers.

**Is enabling differentiation an XX-and-XY-common embryonic function of Dazl?**

The biochemical function of DAZL protein in embryonic germ cell development
is unknown. We speculate that at least one of Dazl's early embryonic functions is
common to XX and XY germ cells, and that Dazl's role in both XX and XY germ cells at
around E12.5 is to enable differentiation.

We consider a common, early function for Dazl in XX and XY germ cells to be
likely, first because the timing of Dazl's expression is about the same in both XX and XY
Fig. 7. Model for the relationship between *Dazl, Stra8, Oct4* and the processes of meiosis initiation and exit from pluripotency in XX germ cells. Note that the arrows represent genetic rather than biochemical relationships.
Meiosis

- Stra8
- Dmc1
- SCP3
- γH2AX

Dazl

- Oct4
- Dppa3/Stella

Pluripotency
germ cells. Second, XX and XY Dazl -/- germ cells appear, judging from the histology, to arrest in the same cell type and at the same time (Fig. 3; Lin and Page, 2005). We know, from previous studies, that this is the time point at which dramatic differentiation events including the loss of ability to form mitotically capable, pluripotent EG cells take place. Although there is at least one sex-specific difference in the phenotype of the Dazl knockout, XY Dazl -/- germ cells die prenatally while most XX Dazl -/- germ cells do not, this could be due to differences in the gonadal environment. For instance, it is known that XX and XY germ cells have different requirements for Bcl-x to survive embryonically, most likely due to differences in the XX and XY gonadal environment (Kasai et al., 2003a). Thus, despite this sex difference, the early function of Dazl might be common to XX and XY germ cells.

Reports that human DAZ and DAZL proteins bind human PUM2 protein raised speculations that DAZ family genes might be involved in the switch between stem cell and differentiated cell in the germ line (Moore et al., 2003). PUM2 belongs to a family of translational regulators that may have an evolutionarily conserved role in regulating the transition from mitosis to differentiation and/or meiosis (Crittenden et al., 2002; Crittenden et al., 2003). For example Pumilio in D. melanogaster is required for maintenance of germ line stem cells (Lin and Spradling, 1997). In C. elegans, FBF-1 and FBF-2 proteins promote mitosis and self-renewal of germ line stem cells in hemaphrodites (Crittenden et al., 2003), while another member of the family, PUF8, promotes meiotic differentiation (Subramaniam and Seydoux, 2003).

We propose that mouse DAZL functions, in embryonic germ cells at around E12.5 to enable differentiation- defined as the ability to respond to signals that regulate
loss of pluripotency, entry into meiosis and chromatin restructuring to reflect sexual identity (this paper; Lin and Page, 2005).

Conclusion

C57BL/6 represents a reproducible and widely studied background on which a previously unsuspected function for Dazl in the transition of XX germ cells from pluripotent gonocyte to terminally differentiating oocyte can now be further examined. The Dazl knockout also provides a starting point to study sexually dimorphic germ cell differentiation and competence for meiotic initiation. The ability to undergo meiosis is a defining feature of germ cells, and studying how the transition from a pluripotent cell type to a cell type capable of meiosis and differentiation is regulated should shed light on the nature of germ cells.
Materials and Methods

Backcrossing $Dazl^{TM1Hg}$ onto C57BL/6 strain background

Mice carrying the $Dazl^{TM1Hg}$ allele were a gift from Howard Cooke, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK (Ruggiu et al., 1997). We crossed $Dazl^{TM1Hg/+}$ mice to C57BL/6 mice (Taconic Farms Inc., Germantown, NY). All the mice are descended from at least one C57BL/6 male and one C57BL/6 female. All experiments were carried out on mice for which $7 \leq N \leq 11$, and 99.2% to 99.9% of the genome is expected to be of C57BL/6 origin.

Embryo Dissection/ PCR-based Genotyping

Timed matings were performed. Noon on the day a vaginal plug was found was designated as E0.5. Collecting and processing of embryos for in situ hybridization and PCR determination of sex of E11.5 embryos were as previously published (Menke et al., 2003). Genotyping PCR was carried out also as previously described (Ruggiu et al., 1997).

Histology and Immunofluorescence

For histological studies, gonadal tissues were fixed in Bouins overnight at 4°C. For immunohistochemistry, gonadal tissues were fixed in 4% paraformaldehyde, either overnight at 4°C, or 4 hours at room temperature. The tissue samples were stored in 70% ethanol and then embedded in paraffin and sectioned. Slides for histological examination were stained with hematoxylin and eosin. Just before use, slides for immunohistochemistry were dewaxed, rehydrated, and autoclaved in 10mM Sodium Citrate for 5 minutes at about 121°C. Rabbit anti-MVH was a gift from Toshiaki Noce, Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan, and it was used as published
(Toyooka et al., 2000). Rabbit anti-SCP3 was a gift from Christa Heyting, Department of Genetics, Agricultural University, Wageningen, The Netherlands, and it was used at a 1:700 dilution (Lammers et al., 1994). Rabbit monoclonal anti-γH2AX (PH3, Upstate Biotech. #07-145) was used at a 1:1000 dilution. Texas Red-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc.) was used at a dilution of 1:200.

**In Situ Hybridization**

In situ hybridizations on E11.5 to E15.5 gonads and subsequent image capture of stained gonads were carried out as previously described (Menke et al., 2003; Wilkinson and Nieto, 1993). Dazl +/+ and Dazl -/- gonads were processed for whole mount in situ within the same tube to exclude tube-to-tube variation as a factor for differential staining. Digoxigenin riboprobes were generated as previously described (Menke et al., 2003; Menke and Page, 2002). The construction of the Dazl, Oct-4, Dppa3/Stella, Stra8 and Dmc1 probes have been described in previous publications (Menke et al., 2003) (Lin and Page, 2005).

**Acknowledgements**

We thank Min Wu for the Dazl riboprobe; George Enders for the GCNA antibody; Toshiaki Noce for the MVH antisera; Mary Goodheart for animal husbandry; Michael Brown for processing and sectioning gonadal tissue samples; Steve Rozen, Alex Bortvin, Andrew Baltus and Steve Wasserman for advice and comments on the manuscript.
References


Chapter IV

Discussion and Future Directions

A) How does genetic background affect the Dazl phenotype?

B) Is there an alternative model for Dazl's function in embryonic XX germ cells?

C) Is Dazl required for germ cell or gonadal sex determination?

D) Are XX Dazl -/- germ cells functionally pluripotent? Is Dazl required for commitment to gametogenic fate?

E) What is/are Dazl’s role(s) in postnatal germ cell development?

F) Is Dazl a translational regulator?

G) How does Dazl regulate Stra8 and Oct4 mRNA levels?

H) Overview
Here I discuss the implications of my work and outline the questions and experiences that arise from what we now know about an embryonic requirement for \textit{Dazl} in murine germ cell development.

The \textit{Dazl} deficiency allele backcrossed onto a C57BL/6 background reveals an earlier requirement for \textit{Dazl} in germ cells than was previously reported. Both XX and XY germ cells exhibit a developmental arrest around E13.5 (this is approximately two days after \textit{Dazl} RNA is first expressed). This is a critical time in germ cell development, as it is when germ cells transition from a pluripotent state to a sexually dimorphic, differentiated state. Indeed, XX and XY germ cells in \textit{Dazl} mutant mice appear to arrest in a sexually undifferentiated state, as judged by histological examination of germ cell morphology. Additionally, XX germ cells deficient for \textit{Dazl} do not down-regulate markers of pluripotency (\textit{Oct4} and \textit{Dppa3/Stella}) or up-regulate markers of meiotic entry (\textit{Stra8}, \textit{Dmc1}, \textit{γH2AX} and SCP3). However, beyond E14.5, the XX and XY phenotypes in \textit{Dazl} deficient mice diverge. XY \textit{Dazl} \textit{-/-} germ cells begin to die by apoptosis, and are virtually absent by birth. XX \textit{Dazl} \textit{-/-} germ cells persist through birth, but grow abnormally large and are not correctly positioned in the postnatal gonad.

With the characterization of the \textit{Dazl} knockout phenotype on a pure strain background, we are now positioned to address key questions about germ cell development and the biochemical function of DAZL. Specifically, data from this, and other, work leads me to postulate roles for \textit{Dazl} in germ cell and gonadal sex determination, germ cell commitment to a gametogenic fate and the mitosis-meiosis transition in postnatal XY germ cells. Further, with data on the biological processes that require \textit{Dazl}, the next step in elucidating \textit{Dazl}'s function must include biochemical
studies on DAZL protein. Experiments should address whether murine Dazl is indeed a translational regulator and, if so, what its embryonic targets are.

A) How does genetic background affect the Dazl phenotype?

Clearly genetic background has a huge impact on the Dazl knockout phenotype. Specifically, the early functions of Dazl appear to be masked in mixed background mice compared to C57BL/6 mice. It is unclear how wide-spread the phenomena of genetic background having a dramatic impact on mutant phenotype in germ cell development mutants is as studying mutants in a pure-strain background tends to be the exception rather than the rule. However, there are at least two other examples of genetic background masking embryonic germ cell mutant phenotypes. The Pinl mutant mice showed no embryonic germ cell phenotype until backcrossed onto a C57BL/6 background, and the TrkB embryonic germ cell mutant phenotype was only fully penetrant on a C57BL/6 background (Atchison et al., 2003; Spears et al., 2003).

Some strains of mice have loci that compensate for the loss of Dazl. I speculate that this is due to the existence of multiple pathways that regulate the crucial transition between pluripotent and committed, sexually dimorphic germ cells and that these regulatory pathways are of differential importance in different strain backgrounds. The results presented here suggest that Dazl functions in one such regulatory pathway but do not preclude the presence of redundant pathways that are more central in other mouse strains.

However, there are alternative explanations for the genetic background effect. The Dazl loci was not entirely removed by the insertion of the knockout construct (which
removed exons 6 and 7, and part of exon 5- leaving the RRM mostly intact). As assayed by RTPCR, no expression from the knockout allele could be observed in heterozygous mice, so it was assumed that the knockout allele is unstable. Yet the results we observed can be accounted for by the possibility that $\text{Dazl}^{TM1/Hko}$ is not a null allele, and that a partially functional DAZL protein is made in mixed background mice, while none is made in C57BL/6 mice. Conversely, it is also possible that a protein product from the knockout allele that interferes with germ cell development is being expressed in C57BL/6 mice while not in pure strain mice, leading to a more severe phenotype in C57BL/6 mice.

To test this possibility, we should probe for transcript and protein expression from the knockout allele in heterozygote mice (using RTPCR and Westerns) and E13.5 embryonic gonads (via RTPCR and immunohistochemistry). E13.5 is a time point at which $\text{Dazl}$ is highly expressed, but before germ cell loss is observed. It is necessary to carry out Westerns in studying $\text{Dazl}$ heterozygotes since the size of the band produced can distinguish between the knockout and wildtype alleles, while it is technically easier to carry out a section immunohistochemistry on E13.5 gonads than to collect enough for a Western blot.

**B) Are there alternative models for Dazl's function in embryonic XX germ cells?**

In Fig. 7 (page 122), I summarized my hypothesis, based on our current data, regarding the function of $\text{Dazl}$ in embryonic XX germ cells and how it relates to the process of entry into meiosis and exit from a pluripotent state. Essentially, the normal downregulation of $\text{Oct4}$ in $\text{Stra8}$ knockout mice demonstrates that $\text{Oct4}$'s downregulation is $\text{Stra8}$-independent, and the fact that $\text{Oct4}$ is downregulated after $\text{Stra8}$ transcript
appears suggests that *Stra8* upregulation is, in turn, independent of *Oct4*'s disappearance. However, this later relationship is not demonstrated by our data since it is formally possible *Stra8* expression requires the absence of OCT4 protein, or the absence of other regulators of pluripotency (e.g. *Dppa3/Stella*). Hence an alternative model for *Dazl*'s function is that *Dazl* is required for downregulation of OCT4 or *Dppa3/Stella*, while OCT4 or *Dppa3/Stella* in turn suppresses *Stra8* transcript expression. When *Dazl* appears, OCT4 or *Dppa3/Stella* is downregulated and hence *Stra8* is upregulated (Fig. 1).

This model can be tested in several ways. The first, and more painstaking but definitive, is by creating a mouse carrying a germ-cell-specific OCT4 or *Dppa3/Stella*-inducible transgene with an IRES site. With this system we can induce prolonged OCT4 or *Dppa3/Stella* expression in E12.5 embryonic XX germ cells, and examine those gonads for evidence of *Stra8* expression. If the absence of OCT4 or *Dppa3/Stella* is indeed required for the appearance of *Stra8*, then the expected results would be that littermate controls which do not carry the transgene express *Stra8* normally, while OCT4 or *Dppa3/Stella*-induced embryos cannot. However, controls (for instance in situs with *Mvh* probe on transgene-induced gonads) should be done to show that OCT4 or *Dppa3/Stella* induction in these XX germ cells does not cause germ cell loss which can give a false negative with any *Stra8* assay.

Another testable prediction arises from the fact that it has been shown that we can induce *Stra8* expression in embryonic XY gonads as well by culturing them with RA (Jana Koubova, personal communication). If the reduction of OCT4 or *Dppa3/Stella* 3 is required for upregulation of *Stra8*, the prediction would be that RA can also reduce
Fig. 1: Alternative model for the function of Dazl in XX embryonic germ cells and the relationship between exit from a pluripotent state (as represented by downregulation of OCT4 or Dppa3/Stella) and entry into meiosis (as represented by upregulation of Stra8).

In this model, Dazl suppresses OCT4 or Dppa3/Stella levels, while OCT4 protein suppresses Stra8 expression. Hence, prior to E12.5, XX germ cells cannot express Stra8 due to the presence of OCT4 or Dppa3/Stella. At E12.5, with the upregulation of Dazl expression, OCT4 or Dppa3/Stella levels are reduced and hence Stra8 is derepressed and meiosis can proceed.
Dazl → OCT4/ Dppa3/ Stella → Stra8 → Meiosis

Pluripotency
OCT4 or Dppa3/Stella levels. Hence the experiment would be to test RA-cultured XY gonads by immunohistochemistry for the presence of OCT4 or Dppa3/Stella.

C) Is Dazl required for germ cell or gonadal sex determination?

We know that Dazl is required for some sexually dimorphic characteristics that germ cells display: XX and XY Dazl -/- germ cells lack sexually dimorphic differences in 1) nuclear morphology, 2) expression levels of pluripotency genes and 3) expression levels of meiotic genes. These beg the question “Is Dazl required for germ cell sex determination?” To answer this, we must first clearly define sex determination, and then find markers of sexual identity with which to probe the Dazl knockout germ cells.

Two broad considerations affect how we pose the question of Dazl’s role in germ cell sex determination. First, germ cell sex determination is not well defined. The field of germ cell sex determination has, for historical reasons, defined early germ cell sex by whether embryonic germ cells were (morphologically) in meiosis or proliferation arrest. With the discovery of new markers, it is unclear if the sexual identity of a germ cell includes all sexually dimorphic characteristics, or is limited to the ones directly related to meiosis and proliferation arrest. Second, murine germ cell sex is primarily a function of the chromosomal sex of the gonad in which they develop. The question about a requirement for Dazl-- a germ-cell-specific transcript-- in germ cell sex determination must be phrased as “Is Dazl required for germ cells to respond to sexually dimorphic signals from the gonadal environment?”

If the female state is defined as embryonic entry into meiosis and the male state as embryonic proliferation arrest, then it would appear that Dazl -/- germ cells are incapable
of becoming male or female. However, from comparing *Oct4* expression in the XX *Dazl* 
-/- and XX *Stra8* -/- gonads, we know that at least one marker of female-specific
differentiation is independent of *Stra8's*, and hence (we believe) meiosis. The possibility
that XY germ cells undergo differentiation events that are separable from proliferation
arrest has not been well explored. Indeed, we now know that XY *Dazl* -/- germ cells fail
to undergo *de novo* methylation that begins in XY wildtype germ cells at E14.5. This
might be due to the absence of *Dmnt3a*, the enzyme required for *de novo* methylation to
occur, in surviving XY *Dazl* -/- germ cells at this time point (Yueh-Chiang Hu; Personal
communication). Whether this is a phenotype that is tied to their entry into proliferation
arrest is unknown.

Overall the field of embryonic murine germ cell development has focused on
entry into meiosis, as opposed to proliferation arrest, as the sole mark of sex
differentiation. The work discussed here suggests that this is only one aspect of sexually
dimorphic development, and that there are other processes worth studying in the context
of germ cell sex determination.

Assuming a broader definition of germ cell sex, we know that post-E12.5 XX and
XY *Dazl* -/- germ cells have at least one phenotypic difference: XY *Dazl* -/- germ cells
die by apoptosis embryonically, while many XX *Dazl* -/- germ cells survive through
birth. There is some evidence that germ cell survival requirements are dependent on the
sex of their gonadal environment. Both XX and XY germ cells require more *Bcl-x* to
protect them from apoptosis within an embryonic testis, compared to within an
embryonic ovary (Kasai et al., 2003). Hence, in at least this respect, XX and XY *Dazl* -/-
germ cells may respond to sex-specific signals from their somatic environment to
produce a sexually dimorphic phenotype (germ cell death versus germ cell survival). Hence, \textit{Dazl} is not required for all aspects of germ cell sex differentiation, but only a subset of sexually dimorphic characteristics.

The next experimentally accessible step is to define what sexually dimorphic pathways require \textit{Dazl}. This is currently hampered by a paucity of markers. Finding more markers will help us better define sexual identity in germ cells and also assess whether \textit{Dazl} is required for these processes. One method for accomplishing this would be a gene expression profile comparison of post-E12.5 XX, post-E12.5 XY \textit{Dazl} -/- and post-E12.5 wildtype germ cells via microarray analysis. A method of isolating PGCs from embryonic gonads for microarray analysis has been published (Molyneaux et al., 2004).

Two caveats to this sort of analysis are: 1) \textit{Dazl} is expected to be a translational regulator, and the differences in transcript levels are expected to be secondary effects of \textit{Dazl} deficiency; 2) XY \textit{Dazl} -/- germ cells are clearly in apoptosis by E14.5, and it is not clear that any differences even at E12.5 and E13.5 will not be overwhelmed by changes that are directly involved in apoptosis. However, the data should be useful in discerning which pathways lie downstream of \textit{Dazl} at least in XX germ cells, and provide a starting point from which to study murine germ cell sex determination.

An argument can be made for the same analysis on the somatic components of XX \textit{Dazl} -/- gonads. Oocytes are thought to actively organize the somatic ovary to the extent that absence of germ cells in early XX gonads leads to degeneration of the gonad (Matzuk et al., 2002). Specifically, there is evidence that E14.5 oocytes, but not E12.5 oogonia, can 'feminize' the somatic gonad by inhibiting cord formation (Yao et al., 2003).
However, it is unclear if the fact that E14.5 oocytes are in meiosis, while E12.5 oogonia are not, is the crucial distinction. To pose this question, we can compare the expression profile of E14.5 somatic ovaries that have been exposed to XX wildtype, XX $Dazl^{-/-}$ or no germ cells (as in the case with $Wv/Wv$ mice). From this we can find out what changes in the somatic ovary are induced by the presence of wildtype oocytes, and also what subset of these effects require $Dazl$.

D) Are $Dazl^{-/-}$ germ cells functionally pluripotent? Is $Dazl$ required for commitment to gametogenic fate?

I speculate, based on my data, that $Dazl^{-/-}$ germ cells can not transition from having stem cell characteristics (capable of proliferation and multi-lineage differentiation), to a more differentiated, gametogenic cell type (committed to becoming gametes). One way to test this hypothesis is to attempt to make EG cells out of E14.5 XX $Dazl^{-/-}$ germ cells. Wildtype E14.5 germ cells can no longer give rise to EG cells. If $Dazl^{-/-}$ E14.5 germ cells can give rise to EG cell colonies when cultured, it would argue that $Dazl^{-/-}$ germ cells, unlike wildtype germ cells, do not lose their capacity for mitotic proliferation beyond E12.5. To test the multilineage potential of such $Dazl^{-/-}$ E14.5 EG cells, I would inject them into GFP-expressing blastocysts and determine whether the non-fluorescent $Dazl^{-/-}$ cells can contribute to multiple cell types in the embryo. If this proves possible, it would be the most convincing evidence of a requirement for $Dazl$ for germ cells to exit a pluripotent, proliferation-enabled state.
E) What is/are Dazl's role(s) in postnatal germ cell development?

We observe a clear requirement for Dazl in embryonic germ cell development at E12.5, but the question remains if Dazl has functions later in germ cell development. Dazl is expressed highly in postnatal germ cells in both male and female mice. XY germ cells in Dazl -/- mice on a mixed background survive to postnatal time points and exhibit defects then. These data suggest that Dazl is also required for germ cell development postnatally. Characterizing the postnatal function(s) of Dazl would be difficult using C57BL/6 Dazl -/- mice, because germ cells in these mice manifest defects embryonically.

To characterize the postnatal function(s) of Dazl, we can either identify a pure strain background on which Dazl -/- germ cells do not manifest defects until after birth, or construct an inducible knockout allele of Dazl and study the phenotypic effect of loss of Dazl function in postnatal germ cells. I suggest that the latter would be more feasible as I have observed Dazl phenotype in a Balb/c background to be grossly similar to that in a C57BL/6 background (unpublished data). As Balb/c is a Mus musculus domestic strain, while C57BL/6 is a Mus musculus musculus strain, it might be challenging to find a pure strain in which the defect differs significantly.

If an inducible Dazl knockout is made, we can determine whether Dazl is required in XY germ cells, as it is in XX germ cells, for the up-regulation of Stra8 and the down-regulation of Oct4. We would induce loss of Dazl postnatally and assay for Stra8 and Oct4 levels via section in situ hybridization. This is to capture spatial data should we have the hypothesized results that Stra8 disappears and Oct4 increases. Spatial data would help us distinguish between, for example, an increase in Oct4-positive germ cells compared to increased levels of Oct4 in each Oct4-positive cell. We will also be able to
assess whether the meiotic prophase arrest observed in both XX and XY mice in a mixed background can be recapitulated as a later function of \textit{Dazl}.

\textit{F) Is Dazl a translational regulator?}

An important next step in the study of \textit{Dazl} function is to ask what molecular mechanisms link \textit{Dazl} to the processes it is genetically required for. Several pieces of evidence suggest that DAZL is a translational regulator: the RRM of DAZL is evolutionarily conserved, mouse DAZL can bind RNA, and DAZL was found to be associated with ribosomes in sedimentation assays. However, direct proof of murine DAZL's ability to regulate translation is lacking, and (if \textit{Dazl} is a translational regulator) no published attempt has identified \textit{in vivo} RNA targets of DAZL relevant to its embryonic germ cell function(s).

We can answer these queries by carrying out the direct biochemical test of the impact of DAZL expression on the expression level of all proteins in ES cells. The technical challenges of acquiring sufficient E11.5 germ cells to do biochemical experiments leads me to suggest EG cells made from E11.5 germ cells or ES cells as reasonable cell culture approximation of E11.5 germ cells. In this experiment, \textit{Dazl} knockout ES cells are transfected either with a control RRM protein that is not involved in translational regulation, or with a \textit{Dazl} expression construct. Protein is harvested from cultures of these two lines and a genome-wide scan of their protein expression pattern and levels is carried out by running a two-dimensional protein gel. The gels can then be compared between the lines transfected with \textit{Dazl} or with the control RRM. Proteins that are over-expressed or under-expressed specifically in the DAZL-expressing line are
candidates for DAZL translational regulation. mRNA levels of the genes for all
candidate proteins identified compared to the control transfected line. It is possible that
DAZL overexpression can drive ES cells to differentiate or die, affecting protein levels
by affecting cell number or transcription level of those genes, giving us false positives.

I further hypothesize that Dazl releases from repression mRNA which are
translationally repressed by Pum2. Pum2 belongs to the PUF family of proteins that have
been suspected to have an ancestral function in the regulation in stem cells of the decision
between mitotic cycling and differentiation (see the review of functions and expression
pattern of various PUF family members on page 37). Human PUM2 and DAZL protein
have been shown to physically interact (Moore et al., 2003). It has been suggested that
Drosophila boule might function primarily to derepress the translation of twine as twine
mRNA is present at high levels in the absence of boule, yet no trace of its protein is
observed. Taken together, it would be interesting to repeat the 2D gel experiment with
Pum2 knockout ES cells. If Dazl regulates translational upregulation at least partly by
inhibiting Pum2 function, then Pum2 knockout ES cells should show similarities in
protein expression with Dazl over-expressing ES cells.

G) How does Dazl regulate Stra8 and Oct4 mRNA expression?

Genetically, I have defined a requirement for Dazl in C57BL/6 mice for the
upregulation of Stra8 and the downregulation of Oct4. How Dazl regulates these
mRNA's may be uncovered by identification of the direct targets of DAZL translational
regulation, as the experiment outlined in the previous section suggests. However, another
way to answer this question is to try to identify what lies immediately upstream of Stra8 upregulation or Oct4 downregulation and try to link those events to Dazl's functions.

There is evidence that stimulation of Retinoic acid receptors (RARs) is required and sufficient for both XX and XY germ cells at E12.5 to express Stra8 (Jana Koubova, personal communication). However, neither somatic cells in the XX gonad nor migrating PGCs express Stra8, even though both these cell types are exposed to RA. This suggests that other factors in E12.5 germ cells predispose them to express Stra8. One such factor, at least in C57BL/6 mice, is the presence of Dazl.

Assuming that it is the transcription of Stra8 that is being regulated in a Dazl-dependent fashion in XX germ cells, it would be of interest to observe the distribution of RARs in Dazl knockout germ cells. It has been shown, in sertoli cells, that addition of RA induces RARs to translocate into the nucleus where the RARs function to regulate transcription (Braun et al., 2000). Thus, if Dazl enables Stra8 expression by enabling RA to activate RARs, then the immunohistochemistry with anti-RARs should show a difference in RAR localization between Dazl knockout and wildtype XX E13.5 germ cells. The prediction is that in Dazl -/- E13.5 XX germ cells RARs stay cytoplasmic, while in wildtype E13.5 XX germ cells RARs have translocated into the nucleus.

Mechanisms for Oct4 downregulation in pluripotent cell types has been the subject of some interest in the field of ES cells/stem cells as Oct4 is considered a "master regulator" of pluripotency. For instance it has been published that Sox2 (another transcription factor that is pluripotency-associated) and LRH-1 (an orphan nuclear receptor) are required for maintenance of Oct4 transcript in the epiblast of embryos (the part of the embryo from which germ cells are formed). In order to try to connect the
Oct4 transcript levels to DAZL's biochemical function, the first step would be to find out which of these 'Oct4-regulators' are expressed in embryonic XX gonads, and from there, test for expression levels and distribution of these Oct4-regulators in Dazl knockout compared to wildtype XX gonads.

H) Conclusion

The many processes which might involve Dazl have been discussed here as discrete pathways, but it is as likely that these pathways interact and Dazl and/or its targets coordinately regulate two or more pathways. An example of such coordination is carried out in C. elegans germ cells by fbf-1/2. fbf-1/2 regulate both the decision to enter mitosis or meiosis (via translational repression of gld-1) and the decision to undergo spermatogenesis or oogenesis (via translational repression of fem3) in hemaphrodites. In murine germ cells, timing of meiotic initiation has long been a marker of germ cell sex. Dazl is required for the expression of Stra8, the earliest marker of germ cell feminization and a gene required for entry into meiotic prophase. Dazl appears well placed to coordinately regulate the processes of germ cell sex differentiation and the transition from mitosis to meiosis or proliferation arrest.

From the markers we have examined, it appears that Dazl -/- germ cells do not undergo any normal differentiation past E12.5—however, many more markers need to be examined to find out what set of processes that take place at this time point Dazl is required for. Insight into how Dazl regulates the processes involved in the transition from a relatively undifferentiated cell type into a sexually dimorphic germ cell type, and how it relates to the other highly conserved genes of early germ cell differentiation that
are expressed around the same time (e.g. *Mvh* and *Pum2* in both XX and XY germ cells, *Nanos2* in XY germ cells) should further define what constitutes ‘lineage commitment’ in a cell lineage that cycles between totipotence (zygote) and terminal differentiation (gamete).

References


