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Review

Germline development in vertebrates and invertebrates

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Abstract. In all animals information is passed from parent to offspring via the germline, which segregates from the soma early in development and undergoes a complex developmental program to give rise to the adult gametes. Many aspects of germline development have been conserved throughout the animal kingdom. Here we review the unique properties of germ cells, the initial determination of germ cell fates, the maintenance of germ cell identity, the migration of germ cells to the somatic gonadal primordia and the proliferation of germ cells during development invertebrates and invertebrates. Similarities in germline development in such diverse organisms as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis* and *Mus musculus* will be highlighted.

Characteristics of the germline: nuage and germ plasm

Germ cells possess special properties due to their unique role. In contrast to somatic tissues which cease to exist when an organism dies, germ cells link successive generations together and are, in that sense, immortal. Since germ cells ultimately give rise to all the tissues in a developing animal, they must remain pluripotent, while at the same time differentiating into highly specialized gametes. The development of the germline is therefore of great interest in studying both the propagation of species and the differentation of a unique cell type.

The origin and nature of the germline have been studied for over a hundred years, dating back to Owen in 1849 who first suggested that some cells in an animal are put aside to contribute to the development of another individual (reviewed in [1, 2]). It has long been recognized that the continuity of germ cells depends on cytoplasmic factors that are inherited from each generation to the next, based in large part on the observation of distinctive cytoplasmic structures in germ cells. Germ cells in more than 80 animals from at least eight phyla contain a characteristic morphological feature called nuage [2]. Nuage is visualized in the electron microscope as a discrete, dense, fibrous organelle, which is unbounded by a membrane, usually associated with clusters of mitochondria, and located in the perinuclear cytoplasm. It is found in germ cells in many stages of development, ranging from primordial germ cells (PGCs) in embryos to gametes in adult gonads. In many animals nuage has been shown to contain RNA and protein, and nuage is widely believed to carry the determinants of the germline.

In many organisms germ cell differentiation depends on a specialized region of cytoplasm in the embryo termed germ plasm, which closely resembles nuage in morphology and ultrastructure. In *Drosophila*, *Caenorhabditis elegans* and *Xenopus*, germ plasm is assembled from germline-specific factors that are deposited in the egg during oogenesis. It is localized to a specific region of the embryo and determines where the PGCs will form

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[1, 2]. Germ plasm is distinguished in all cases by the presence of specialized organelles called germinal granules. Like nuage, germinal granules are not surrounded by a membrane but are visible in the electron microscope as electron-dense, fibrous particles. They are associated with mitochondria and contain many RNAs and proteins. In most animals these granules are localized exclusively to the germ plasm and incorporated into PGCs as they form.

In *Drosophila* several lines of evidence indicate that the germ plasm contains the determinants of the germline. First, transplanted germ plasm can induce PGC formation at ectopic sites [3, 4]. Second, mutations that disrupt germ plasm assembly also prevent germ cell formation (reviewed in [5]). Third, mislocalization of the germ plasm component *oskar* to an ectopic location (the anterior of the egg, instead of the posterior) leads to the formation of ectopic germ cells at the same location [6]. Thus, it is clear that in *Drosophila* the germ plasm contains the determinants of the germline. Since germ plasm in other organisms resembles that in *Drosophila*, this function of germ plasm is likely to be conserved. Indeed, transplantation experiments have indicated a similar role for germ plasm in amphibians [7, 8].

One of the outstanding questions in germ cell development concerns the relationship between germ plasm and nuage. These structures are clearly related since they share many characteristics, including morphology, association with mitochondria, presence of RNA and protein and localization to the germline; these observations suggest that germ plasm and nuage represent different forms of the same material. Support for this idea has come from *Drosophila*, where Vasa protein has been identified as a component of both germinal granules (in the developing oocyte and in the embryo) and nuage (in the nurse cells in the ovary), indicating a direct link between these structures [9, 10] (see below). This finding suggests that nuage may represent a precursor for the germinal granules, although many granule components have been identified that are not found in nuage. The *Xenopus vasa*-like protein, XVLG1, is also found in a juxtanuclear location in embryonic and adult germ cells, implying that it may also be associated with nuage [11]. However, the universality of these findings remains unclear, since in most organisms studies of these structures have been purely descriptive. In particular, mammals do not contain a recognizable germ plasm, even though germ cell-specific nuage has been well characterized in many mammalian species. Thus, further analysis of the molecular nature of these structures will be required to gain a better understanding of their function.

Determination of the germline

Drosophila

In *Drosophila* the early embryo undergoes a series of rapid, synchronous nuclear divisions that form a syncytium of nuclei. Approximately 10 PGCs (called pole cells in *Drosophila*) are formed at the posterior of the embryo 90–120 min after fertilization, before the somatic nuclei cellularize [12]. Pole cell formation depends on the presence of germ plasm (termed pole plasm), which is assembled at the posterior of the egg during oogenesis and contains germinal granules ('polar granules' in *Drosophila*; fig. 1A). The morphology, assembly and localization of *Drosophila* pole plasm during oogenesis have been well characterized and are reviewed extensively elsewhere [5, 13]. In brief, many components of the pole plasm have been identified using maternal-effect screens for genes affecting early development. At least a dozen genes required for the formation of the pole plasm have been isolated so far [5, 14]. For most of these genes, embryos from homozygous mutant females (referred to hereafter as mutant embryos) do not form germ cells. This leads to a 'grandchildless' phenotype, as mutant females produce sterile progeny. In addition, these mutants do not form abdomen, since they do not localize *nanos* (*nos*) RNA to the pole plasm [15]. Although *nos* is required for abdominal patterning in the embryo and not pole cell formation, it is incorporated into pole cells and plays a role in later stages of pole cell development (discussed below). Assembly of the pole plasm involves the sequential localization of several RNAs and proteins. Various factors arrive at the posterior pole of the egg at different stages of oogenesis, and the proper localization of some components depends on the prior localization of others.

Two of the earliest factors to arrive at the posterior pole are Staufen (Stau) protein and *oskar* (*osk*) RNA, which arrive there together during stages 8–9 of oogenesis [16–18]. Stau possesses a double-stranded RNA-localization motif and is required for posterior *osk* RNA localization; it may be required to maintain the localization of other pole plasm components at the posterior as well. However, Stau localization to the posterior is transient, since Stau is not maintained in the pole plasm during embryogenesis. *osk* is also found in polar granules and plays a critical role in the assembly of the pole plasm, as it is required for the posterior localization of the pole plasm components *vasa* (*vas*), *tudor* (*tud*) and *nos* [5]. Moreover, mislocalization of *osk* to the anterior pole, achieved by replacing its 3′ untranslated region (UTR) (which contains sequences required for RNA localization) with the 3′ UTR from the anteriorly localized *bicoid* RNA, leads to the formation of functional pole cells at the same site [6]. Both *vas* and *tud* are required for pole cell formation at the anterior, and

polar granules, mtlrRNA, *nos* RNA and Nos protein are all found at the anterior in these experiments [6, 19, 20]. These results show that *osk* can drive pole plasm assembly at an ectopic site. Furthermore, they indicate that *osk* localization to the proper site in the developing egg is critical for germline development.

Both *osk* mRNA and protein are localized to the posterior of the developing egg during oogenesis. *osk* local-

ization requires the activities of several maternal effect genes that act upstream of *osk*, including *stau* and *mago nashi* [16, 17, 21]. *osk* RNA localization can be broken into several steps. First, *osk* RNA is transported from the nurse cells, where it is synthesized, to the oocyte. Localization of *osk* to the oocyte requires microtubules, since it is sensitive to microtubule-assembly inhibitors such as colchicine, and *osk* RNA does not accumulate
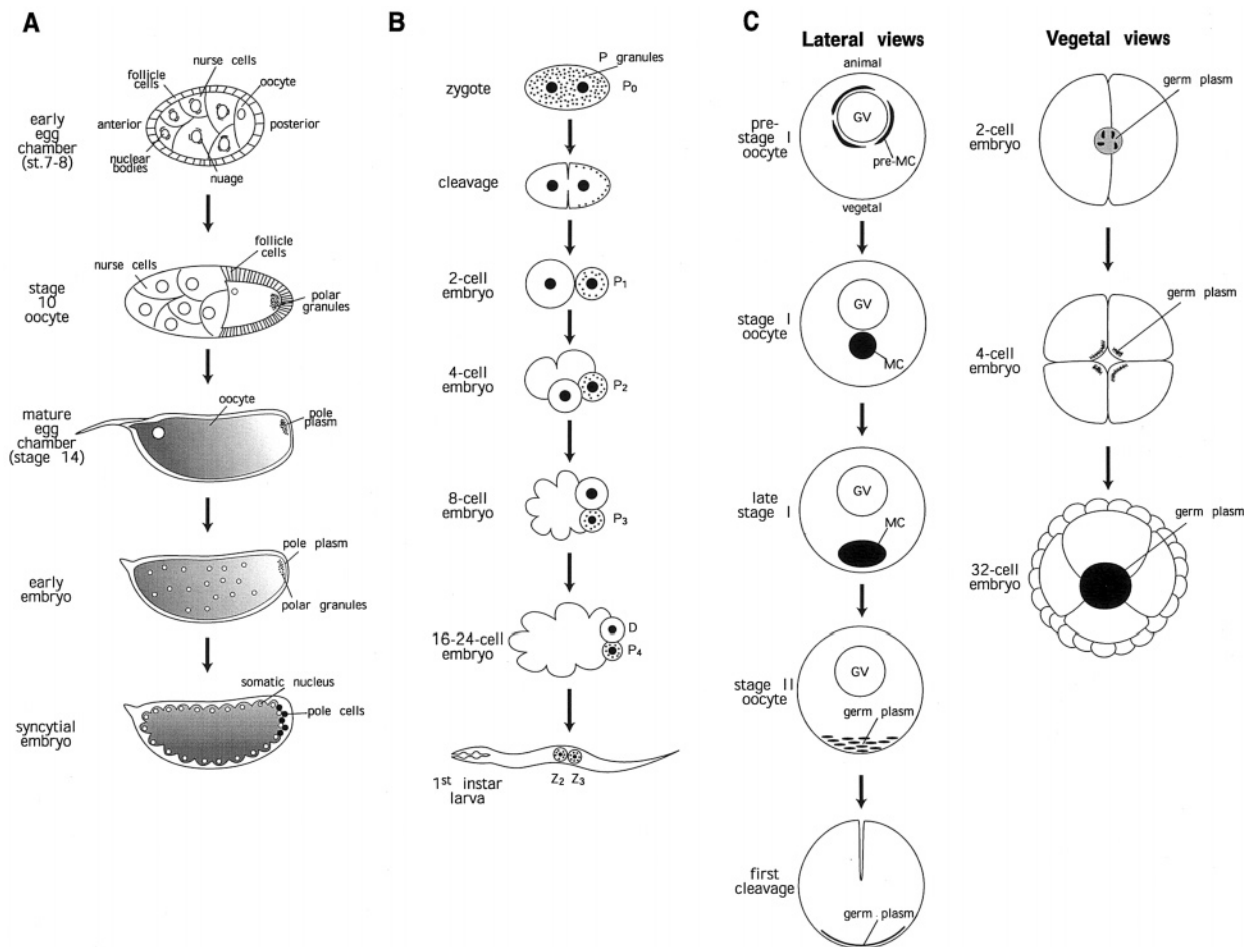

Figure 1. Determination of the germline in *Drosophila* (*A*), *C. elegans* (*B*) and *Xenopus* (*C*). (*A*) Summary of the steps involved in *Drosophila* pole plasm assembly. *Drosophila* oogenesis is divided into 14 stages (reviewed in [201]). A developing egg chamber consists of 16 germline cells (15 nurse cells and 1 oocyte) surrounded by a sheath of somatic follicle cells. As oogenesis proceeds, the oocyte increases in size and the nurse cells transport RNAs and proteins into the oocyte; later the nurse cells degenerate. The germ (or pole) plasm is assembled at the posterior of the oocyte and determines where the primordial germ cells, or pole cells, will form in the embryo. Pole cells form in the syncytial embryo after the nuclei have divided and migrated to the periphery. Posterior nuclei enter the pole plasm and bud off to form pole cells, before the somatic nuclei cellularize. Adapted from [5]. (*B*) Summary of early germline development in *C. elegans*. Embryos and larva are oriented anterior left and ventral down. In the early embryo the germline is segregated from somatic cell lineages via a series of unequal cell divisions; P_1, P_2, P_3 and P_4 represent the germline blastomeres. Germinal (or P) granules are initially distributed throughout the cytoplasm of the zygote but become localized to the posterior during cleavage and are inherited only by the germline blastomeres at each division. The primordial germ cell P_4 divides only once during embryogenesis to form Z_2 and Z_3, the founders of the adult germline; its somatic sister cell, D, gives rise to body muscle cells. Adapted from [82]. (*C*) Summary of the steps involved in *Xenopus* germ plasm assembly. GV, germinal vesicle; MC, mitochondrial cloud. The MC evolves from multiple aggregates surrounding the GV (pre-MC) into a spherical structure which translocates to the vegetal pole, before fragmenting into hundreds of islands. Ultimately 4 vegetal-pole blastomeres in the 32-cell embryo inherit the germ plasm to become the founders of the germline. Vegetal views adapted from [67, 104].

in the oocyte in *Bicaudal-D* and *egalitarian* mutants that disrupt the microtubule organizing center in the oocyte [22, 23]. Furthermore, the microtubule motor protein dynein accumulates in the oocyte similarly to *osk*, and could be involved in transporting RNAs and proteins into the oocyte [24]. Next, *osk* RNA is transported within the oocyte to the posterior pole and anchored there. Both microtubules and several actin-binding proteins, including tropomyosin II, profilin and formin, are required for translocation and maintenance of *osk* RNA at the posterior [23, 25–30]. *osk* RNA could be localized to the posterior by motor-dependent directional transport along the microtubule network, since a fusion protein composed of a portion of the plus end-directed microtubule motor kinesin linked to a reporter gene localizes to the posterior of the oocyte at the same time as *osk* [25, 30]. This motor protein also shows the same genetic and cytoskeletal requirements for localization as *osk*, suggesting that they use similar mechanisms of localization.

An alternative, or perhaps complementary, mechanism for *osk* RNA localization involves the rapid cytoplasmic streaming which occurs in oocytes. In this model, RNAs are transported to the posterior by bulk cytoplasmic flows and then concentrated there by binding to specific anchors. Recent studies have shown that cytoplasmic streaming is capable of localizing RNAs to the posterior, since fluorescently labeled *osk* mRNA injected into developing oocytes at late stages is concentrated at the posterior in a process that requires cytoplasmic flow [31]. However the localization of exogenous RNA in these experiments may not parallel that of endogenous transcripts, since injections were only performed after the stage when endogenous *osk* is normally localized. In addition, cytoplasmic streaming normally occurs after *osk* localization to the posterior [32]. Induction of premature cytoplasmic streaming, by either treating with the actin inhibitor cytochalasin D or mutating one of several actin-binding proteins, leads to a failure to localize *osk*, presumably because *osk* is not anchored at this time [26, 27, 29]. Cytoplasmic streaming may therefore function primarily to localize other RNAs, such as *nos* and *germ cell-less*, at later stages of oogenesis.

Since *osk* RNA must be transported across the oocyte to reach the posterior, its translation must be regulated during the localization process to prevent *osk* from being activated prematurely, before it has reached the posterior. Indeed, Osk protein is not produced in mutants which abrogate *osk* RNA localization, indicating that Osk activity requires localization of its RNA [33, 34]. The mechanism of *osk* translational regulation is complex and not completely understood. Two isoforms of Osk protein, Short and Long Osk, are generated by alternative start codon usage; Short Osk is the major Osk species and is sufficient to induce pole cell and abdomen formation [33, 35]. Repression of premature *osk* translation is mediated by repeated, conserved sequences in the 3′ UTR termed Bruno response elements (BREs); transcripts with the BREs deleted are translated in the oocyte before localization, leading to severe developmental defects [36]. BRE-mediated repression requires at least two proteins, Bruno and p50, that bind the BRE independently [36–38]. Translational repression of *osk* also requires *Bicaudal-C* (*Bic-C*), as *osk* is prematurely translated and Osk protein does not accumulate at the posterior in *Bic-C⁻* ovaries [39]. *Bic-C* encodes an RNA-binding protein of the KH type and could therefore interact directly with *osk* mRNA. Sequences required for translational derepression at the posterior have also been identified in the 5′ UTR of *osk* [38]. Transcripts lacking this derepression element are localized but not translated, indicating that localization alone is not sufficient for *osk* activation. Instead, *osk* translation may depend on posteriorly localized factors that bind the 5′ UTR and, by an active process, overcome BRE-mediated repression. The *vas*, *stau*, *tud* and *aubergine* gene functions, in addition to functional Osk protein, are required for accumulation of wild-type levels of Osk protein [33–36, 40, 41]. Current evidence suggests that *vas*, *stau* and *tud* enhance translation rather than stabilization of Short Osk. *vas* is also required for phosphorylation of Short Osk.

Like *osk*, *nos* RNA is translated only if it resides at the posterior pole [42]. However *nos* appears to be regulated by a different mechanism since, in contrast to *osk*, a 184-nucleotide element in the 3′ UTR is both necessary and sufficient for the localization of *nos* RNA, the repression of unlocalized *nos* RNA and the activation of *nos* at the posterior [43]. These findings suggest that all three processes are tightly linked for *nos*, although a discrete 90-nucleotide element has been shown to mediate repression of unlocalized *nos* RNA independently of the localization signal, indicating that these processes are separable [44, 45]. Repression of unlocalized *nos* RNA depends on Smaug protein, which binds the 3′ UTR [46]. Activation of *nos* at the posterior requires Vas, although no direct interaction between Vas and *nos* RNA has yet been demonstrated [44]. These results indicate that more than one mechanism of translational regulation is employed in the pole plasm. Furthermore, Nos is itself a translational regulator, as it directs abdomen formation in embryos by repressing translation of *hunchback* RNA in concert with Pumilio [47–51]. Unlike *osk* and *nos*, *hunchback* translation is repressed by deadenylation of the messenger RNA (mRNA), which Nos and Pumilio promote [37, 44, 52]. Short poly(A) tracts are also associated with translational repression for many maternal mRNAs in mouse and *Xenopus* oocytes [53–55].

Some pole plasm components are restricted to the posterior via other mechanisms. Vas protein is detected in the germline throughout development: it is incorporated into pole cells as they form, expressed zygotically in migrating pole cells, found in the embryonic gonads and expressed in the germline during oogenesis [9, 10, 56]. Early in oogenesis Vas protein is found in the perinuclear nuage in the nurse cells, and during mid-oogenesis it begins to accumulate at the posterior pole of the oocyte, where it is a component of the polar granules. These observations suggest that Vas may be transported to the posterior of the oocyte as part of the nuage, which is likely to represent the precursor to the polar granules. In support of this idea, it has recently been reported that nuage in *Drosophila* is surrounded by cytoplasmic structures called sponge bodies [57]. These structures were identified as amorphous, electron-dense masses that are associated with mitochondria and rich in RNA and the Exuperantia protein. Sponge bodies are found in the cytoplasm of both nurse cells and developing oocytes and in ring canals, the cytoplasmic bridges through which nurse cells transport materials to the oocyte, implying that sponge bodies may facilitate the localization of germ plasm components such as Vas. In this regard sponge bodies may resemble the mitochondrial cloud (MC) in *Xenopus*, although sponge bodies are not associated with microtubules like the MC (discussed below).

Localization of Vas protein at the posterior also requires localized Osk protein, which may function to anchor Vas at the posterior [10, 58]. Indeed, Osk may facilitate pole plasm assembly in this way, by anchoring nuage-derived germinal material, along with other pole plasm components, at the posterior. Several lines of evidence support this hypothesis. First, Osk protein can interact directly with Vas in vitro, and mutations that weaken this interaction also abolish pole cell formation in vivo, suggesting that Osk drives pole plasm assembly by binding directly to Vas [59]. Second, Osk protein is found in polar granules along with Vas and Tud proteins [9, 10, 20, 59, 60], and is required for Tud and Nos protein localization, in addition to Vas [5]. Third, Osk is required to maintain the localization of its own RNA at the posterior, and may do so in part by anchoring Stau protein, which is also required for *osk* RNA localization; Stau protein is not maintained at the posterior pole in *osk* mutants and has been shown to interact directly with Osk in vitro [34, 59]. Thus Osk binds directly to several pole plasm components, and is required to maintain their presence in the pole plasm. This function of *osk* may be required only in insects with meroistic ovaries, since unlike several other pole plasm components (see table 1), no *osk* homologue in organisms other than *Drosophila* species has yet been identified.

In addition to *nos*, many other pole plasm components are themselves involved in the translational regulation of other germline factors. Vas encodes an RNA helicase of the DEAD-box family, which includes the translation factor eIF4A, and has been shown to possess adenosine triphosphate (ATP)-dependent RNA helicase activity in vitro [56, 60, 61]. During oogenesis Vas is required for translation of *gurken* mRNA, a factor involved in the establishment of polarity in the oocyte [62–64]. Both translation and post-translational modification of Osk also appear to be affected in *vas* mutants [33, 34], and Vas is required for efficient translation of *nos* [44]. These results indicate that Vas acts as a translational regulator in the oocyte, although there is no evidence for a direct interaction between Vas and any of these mRNAs. Given the pleiotropy of *vas* phenotypes [62, 63], it is likely that many Vas target RNAs have not yet been identified. Alternatively, since Vas has not yet been shown to interact specifically with RNA, it may regulate translation through protein-protein interactions with other factors. In support of this idea, Vas binds the Bruno repressor protein directly in vitro [37], and interacts with Osk in the yeast two-hybrid system [59].

Several lines of evidence suggest that translational regulation represents a fundamental and widely conserved feature of germline development. First, *vas* homologues have been found in a diverse range of organisms (see table 1). In each case, *vas* homologues, like *Drosophila vas*, are specifically expressed in germ cells. Moreover, germ-line RNA helicase (GLH) proteins in *C. elegans* are found in germinal granules, and Vas protein in mice is associated with a perinuclear granule in testicular germ cells that may represent nuage [65, 66]. In *C. elegans* and *Xenopus* a role for *vas* homologues in germline development has been demonstrated [66, 67]. These findings strongly suggest that *vas* function in germline development is highly conserved. The translational regulator *nos* is also conserved (see table 1). In *C. elegans* three *nos* homologues have been identified, and two of them are known to be required for PGC development (K. Subramaniam and G. Seydoux, personal communication). *Xenopus Xcat-2* possesses a zinc finger domain similar to that found in *nos*, and is colocalized to the germ plasm, like *Drosophila nos* [68]. Finally, several other pole plasm components in *Drosophila*, such as mtlrRNA (mitochondrially encoded large ribosomal RNA), in addition to germline factors in other organisms, have been implicated in translational regulation; these will be discussed later.

While pole plasm assembly has been well characterized in *Drosophila*, the signal that induces cellularization of the germline, rather than just assembly of the germ plasm, remains unknown. Candidates for this signal include mtlrRNA and *germ cell-less* (*gcl*) RNA, two

Table 1. Conservation of selected *Drosophila* genes involved in germline development.

Name	Molecular nature	Function in *Drosophila*	Homologues*	References
vasa	DEAD-box RNA helicase; translational regulator	PGC determination	*Caenorhabditis elegans* [*glh-1, glh-2, glh-3*]	66, 91
			Xenopus laevis [*XVLG1*]	109
			Danio rerio (zebrafish)	218, 219
			Mus musculus [*Mvh*]	65
			Rattus norvegicus	220
nanos	CCHC zinc finger RNA Binding domains; translational regulator	abdominal patterning PGC migration stem cell development	*Caenorhabditis elegans* (3 genes)	K. Subramaniam and G. Seydoux, pers. comm.
			Xenopus laevis [*Xcat-2*]	107
			Musca domestica (housefly)	221
			Chironomus samoensis (midge)	221
			Helobdella robusta (leech)	222
mago nashi	novel	germ plasm assembly	*Caenorhabditis elegans*†	223, 224
			Xenopus laevis	223
			Mus musculus	223, 224
			Homo sapiens	224
bruno	RNP-type RNA binding domains; translational regulator	anteroposterior patterning gametogenesis	*Caenorhabditis elegans* [*etr-1*]	37
			Xenopus laevis [*etr-1*]	225
			Homo sapiens [CUG-BP]	226
pumilio	novel RNA binding domains; translational regulator	abdominal patterning stem cell development	*Caenorhabditis elegans* [FBF]‡	227, 228
			Saccharomyces cerevisiae	227, 228
			Schizosaccharomyces pombe	227, 228
			Mus musculus	228
			Homo sapiens§	227, 228
boule	RNP-type RNA binding domain; novel 'DAZ' repeats	meiosis	*Xenopus laevis* [*Xdazl*]	210
			Mus musculus [*Dazla*]	209
			Homo sapiens [*DAZ, Dazla*]	205–208
staufen	double-stranded RNA binding protein	RNA localization in ovaries and neural precursors	*Homo sapiens*‖	229
tudor	novel 'tudor domain' repeats	PGC determination	none, but 'tudor domain' protein in *Homo sapiens*	230

Abbreviations are as follows: PGC, primordial germ cell; RNP, ribonucleoprotein. Gene and protein names are given in square brackets.
* In most cases, homologues have been identified based on sequence similarities. Except where discussed in the text or noted here, there is little evidence regarding conservation of function in the germline.
† A *Drosophila mago nashi* mutant can be rescued by introduction of a *Caenorhabditis elegans mago nashi* homologue [223].
‡ FBF acts in the hermaphrodite germline to regulate the switch from sperm to oocyte production, and represses *fem-3* expression via binding to the 3′ UTR of the *fem-3* mRNA [227].
§ A human *pumilio* homologue can bind the same sequences (*nanos* recognition elements, or NREs) as *Drosophila pumilio* in vitro [228].
‖ In *Drosophila*, *staufen* function is not restricted to the germline. It is not known where the human homologue acts.

components of the pole plasm that are not required for its formation. Two lines of evidence suggest that mtlrRNA is required for pole cell formation. First, it can rescue the ability of ultraviolet (UV)-irradiated embryos to form pole cells [69]. Second, embryos with reduced amounts of mtlrRNA in the germ plasm (formed by

injection of anti-mtlrRNA ribozymes) are unable to form pole cells, even though pole plasm is assembled normally, as judged by the correct localization of *osk* and *gcl* mRNAs and Vas and Tud proteins [70]. As mtlrRNA is localized to polar granules [71], it may play a role in the translation of proteins required for pole cell formation, although it has not been determined whether the ribosomes associated with polar granules are mitochondrially derived. The possibility that mitochondrial factors are involved in producing cellular proteins that function in germline specification is not limited to *Drosophila*, since mtlrRNA is also present in germinal granules in *Xenopus* embryos from the four-cell stage to blastula [72].

In addition to mtlrRNA, *gcl* also appears to be required for pole cell formation, since reduced levels of *gcl* RNA during oogenesis (achieved by expressing antisense *gcl*) lead to the formation of fewer pole cells, while increased levels of *gcl* expression lead to increased numbers of pole cells [73]. Gcl protein is associated specifically with the nuclear pores of germ cell nuclei [74]. Neither mtlrRNA nor *gcl* is sufficient to induce pole cell formation at an ectopic site, however, indicating that multiple factors are required for this process. It is likely that additional factors involved in inducing pole cell formation have not yet been identified.

C. elegans

The germline in *C. elegans* is segregated from somatic lineages early in embryogenesis, from the time of the first division of the fertilized egg (reviewed in [75, 76]). The primordial germ cell (P_4) is formed by a series of four asymmetric, stem cell-like divisions of the zygote (P_0; fig. 1B). Each asymmetric cleavage forms a large somatic blastomere and a smaller germline blastomere, or P cell (P_1, P_2, P_3, and P_4); at each division, germinal granules (termed P granules) are partitioned solely to the P cell. P_4 divides only once during embryogenesis, at about the 100-cell stage. It divides equally and distributes P granules to both its daughters, Z_2 and Z_3. These cells do not divide until after hatching of the larva, at which point they undergo extensive proliferation to give rise to approximately 1500 germ cells in the adult hermaphrodite. P granules are found associated with the nuclei of all germ cells throughout larval and adult development (except sperm), and are passed on to offspring via the oocyte. Thus P granules effectively mark the germline throughout development.

Analysis of *C. elegans* P granules has centered on two experimental approaches: the use of P granule-specific antibodies as markers, and the generation of maternal effect mutations that disrupt P granule localization. P granule segregation during the early cleavages has been followed in fixed embryos [77, 78], and in live embryos using laser-scanning confocal microscopy to visualize fluorescently labeled anti-P-granule antibody [79]. Initially P granules are distributed uniformly in the cytoplasm of the egg. Shortly after fertilization a general flow of cytoplasm towards the posterior pole occurs, carrying the P granules to the posterior. P granules are then anchored to the posterior cortex, as they do not cycle back to the anterior with other cytoplasmic components. The first embryonic division occurs along the anteroposterior axis, and is positioned such that the posterior cell, the germline blastomere P_1, inherits the P granules. P granules at the anterior disappear during mitosis, and none are visible in the anterior, somatic daughter cell; the destabilization of P granules in somatic cytoplasm is not well understood, but most likely mislocalized granules are either degraded or disassembled. In the P_1 cell P granules also localize to the posterior, in an apparently similar mechanism of cytoplasmic localization and cortical attachment, but they appear to be localized via a different mechanism in P_2 and P_3. In these cells the polarity of division is reversed, and the daughter P cell is formed to the anterior of its sister. P granules first become perinuclear, and are then moved with the nucleus toward the site of formation of the next P cell.

As in *Drosophila*, the cytoskeleton plays a key role in localizing germ granules in *C. elegans*. In P_0 and P_2 cells, both P granule localization and depletion of stray P granules require actin microfilaments, while the ventral movement of the nucleus in P_2 requires microtubules in addition to microfilaments [78, 79]. The processes of P granule localization and asymmetric cell division are clearly linked, as cytoskeletal inhibitors that disrupt the former process also disrupt the polarity of P-cell divisions [80, 81]. Indeed, the actin cytoskeleton is required for the alignment of the mitotic apparatus along the axis of P granule segregation in P_0. Many mutations that affect P granule segregation (e.g. *mes-1*, *par*) also show specific effects on centrosome positioning and alignment of the mitotic apparatus [81].

A number of genes required for the proper partitioning of P granules have been identified, including *maternal-effect sterile 1* (*mes-1*) and the *partitioning defective* (*par*) genes. In *mes-1* embryos, the P_4 cell is transformed into a muscle precursor, like its somatic sister cell ('D'), and generates up to 20 body muscle cells instead of germ cells [82]. This change in cell fate is due to defects in the asymmetric cell divisions that produce P_4. P_2 (in some embryos) and P_3 (in most embryos) divide in the wrong orientation and missegregate P granules into both daughter cells. Moreover, in most *mes-1* embryos P_4 divides precociously and undergoes at least one extra round of division, and no Z_2/Z_3 cells are present in L1. Proper segregation of P granules also requires the *par* family of maternal-effect genes, which

is required to establish polarity in the early embryo. Mutations in any of six *par* genes disrupt anterior-posterior asymmetries and lead to abnormal patterns of cell fate [83, 84]. In embryos from *par* mothers the P_0 zygote undergoes a symmetric first division to produce two equal cells; these daughter cells show aberrant cell cycle length and misaligned mitotic spindles, and defects are seen in all subsequent cell divisions. *par* mutants generally show missegregation or no segregation of a number of cytoplasmic determinants of cell fate, including P granules. The PAR proteins examined so far are all enriched at the cortex of the cytoplasm in P_0, and in some cases polarized along the anterior-posterior axis: PAR-1 and PAR-2 become localized to the posterior of the fertilized egg, whereas PAR-3 concentrates at the anterior and PAR-4 is distributed uniformly along the anterior-posterior axis [85–88]. The asymmetric localization of PAR-1, PAR-2 and PAR-3 is reestablished in each cell cycle, suggesting that these proteins are also required for asymmetric divisions and P-granule partitioning in P_1, P_2 and P_3. The *par* genes are unlikely to function in a single pathway since their phenotypes differ in their effects on the localization of P granules and somatic cell fate determinants (such as *glp-1*), and on orientation of the mitotic spindle [81, 88]. PAR-1 possesses a C-terminal domain that interacts with a nonmuscle conventional myosin which is itself required for embryonic polarity [89], suggesting that it may interact with the cytoskeleton.

P granules hybridize to oligo(dT) and therefore contain polyadenylated RNAs [90], but no RNA component of P granules has yet been identified. The first P granule proteins to be identified were the putative germ-line RNA helicases (GLHs). At least three *glh* genes have been identified in *C. elegans* so far, and all three predicted proteins possess a DEAD-box helicase motif and are similar to *Drosophila* Vas [66, 91]. Like *vas*, *glh-1* and *glh-2*, but not *glh-3*, also encode a set of amino-terminal glycine-rich repeats. GLH-1 and GLH-2 contain four and six CCHC zinc fingers, respectively, of the type found in the RNA-binding nucleocapsid proteins of retroviruses; GLH-3 possesses two divergent zinc fingers of the same type. Like Vas, GLH-1 and GLH-2 proteins localize to P granules at all stages of development and are required for adult fertility, as demonstrated by the injection of antisense *glh-1* or *glh-2* RNA [66]. Sterile *glh-1* or *glh-2* worms have underproliferated germlines and altered nuclear morphology in germ cells, implying that *glh* genes function in postembryonic germline development. Injection of antisense *glh-1* or *glh-2* also leads to the absence of several P-granule epitopes (including *pgl-1*; see below), suggesting that these genes play a role in the assembly of P granules. Another P granule component, *pgl-1*, has recently been identified in a screen for mutants failing to stain with an anti-P-granule monoclonal antibody [92]. *pgl-1* encodes a novel protein with an RGG-box motif found in a number of RNA-binding proteins, and is associated with P granules at all stages of development. *pgl-1* mutants are sterile due to reduced germline proliferation and defects in gametogenesis. In addition they have defective P granules, as judged by the absence of several P granule epitopes. In sterile *glh-1* mutants *pgl-1* loses its association with P granules and is found in the germline cytoplasm. Since the sterile phenotype of *glh-1* closely resembles that of *pgl-1*, it may result from *pgl-1* dissociation from the P granules, implying that P-granule association is essential for *pgl-1* function.

Four other proteins (PIE-1, MEX-1, POS-1 and MEX-3) have also been found to associate with P granules, but they do so only transiently, during early embryogenesis. *pie-1* is required for transcriptional repression in germline blastomeres, and is associated with P granules in the P cells, including P_4, but not in the daughters of P_4 [93]. PIE-1 is discussed further below. *mex-1* functions in both germline and somatic cell lineages; in the germline, it is required for the proper segregation of P granules during the first embryonic cleavages [94, 95]. In newly fertilized *mex-1* eggs, P granules accumulate at the posterior but are not anchored at the cortex, leading to incomplete localization and, consequently, a loss of P granules in P cells due to their incorporation into somatic blastomeres. Hence germ cells do not form in these mutants. *mex-1* encodes a protein with two copies of a predicted Cys/His finger motif that is also found in PIE-1 [96]. The protein is enriched in germline blastomeres and associated with P granules in each P cell, but not in the daughters of P_4 or in germ cells at later stages. *mex-1* also appears to affect the structure of P granules, since the amount of granule-associated PIE-1 is reduced in *mex-1* mutants. Indeed, inefficient PIE-1 localization to germline blastomeres may contribute to some of the somatic defects seen in *mex-1* mutants. POS-1 resembles MEX-1 in several ways, including protein localization during early embryogenesis and association with P granules, protein sequence (it possesses two copies of the same zinc-finger motif) and phenotype, since it also functions in both germline and somatic cell lineages and is required to form germ cells [97]. In embryos produced by *pos-1* mothers, the P_3 cell shows little or no cleavage asymmetry, in that P granules and PIE-1 are present at equal levels in both its daughters; P_4 adopts the fate of its sister cell as it divides several times and produces muscle cells. Finally, *mex-3* encodes a predicted RNA-binding protein of the KH type and is found in somatic as well as germline blastomeres [98]. Like PIE-1, MEX-1 and POS-1, MEX-3 associates with P granules in germline blastomeres, but not in germ cells in late embryos or in the gonads. *mex-3* appears to play a role in both the proper

segregation of P granules during the third embryonic division and in the proper development of the P_3 cell. The loss of PIE-1, MEX-1, POS-1 and MEX-3 immunoreactivity in P granules after the early cleavage stages suggests that either the composition of P granules changes during early development, or that their structure changes, rendering epitopes for these proteins inaccessible. The relationship between *C. elegans* P granules and *Drosophila* polar granules remains unclear in certain aspects. Mutants for all the known P granule components still contain P granules, in marked contrast to mutants for most *Drosophila* polar granule components which fail to assemble polar granules [5]. Moreover, the mis-segregation of P granules to both P_4 and its sister cell, D, in *mes-1* embryos suggests that P granules are not sufficient to specify germ-cell fate, since D never adopts a germline fate even though it inherits P granules [82]. Perhaps the number of P granules inherited in *mes-1* mutants are insufficient to specify germline fate, or *mes-1* P granules are defective in some way. *mes-1* mutants may resemble *Bicaudal-C* and *Bicaudal-D* mutants in *Drosophila*, which mislocalize *osk* but do not form ectopic pole cells [16, 17, 39, 99].

Xenopus

In anuran amphibians such as *Xenopus laevis* and *Rana pipiens*, germ plasm at the vegetal pole of the egg is incorporated into germline cells during embryogenesis and believed to act as the determinant of the germline [2]. In early *Xenopus* oocytes germinal material is contained in a structure called the mitochondrial cloud (MC; also known as Balbiani's body), which has been well studied for over 25 years [100–102]. Early studies identified the MC as a distinctive mass in the cytoplasm of previtellogenic oocytes that grows rapidly and is rich in mitochondria and electron-dense granulofibrillar material (GFM). In pre-stage I oocytes, multiple mitochondrial aggregates (premitochondrial clouds) surround the germinal vesicle (fig. 1C). These evolve into a spherical structure located at the ventral side of the germinal vesicle, then fragment into hundreds of islands containing mitochondria and GFM. These islands are localized in the peripheral cytoplasm at the vegetal pole of the oocyte. Since GFM closely resembles embryonic germinal granules, these observations suggest that the MC serves to accumulate and localize germinal material in early oocytes [102]. In support of this idea, the MC plays a central role in the localization of germ plasm components to the vegetal pole (discussed below).
After fertilization of the *Xenopus* oocyte, the islands of mitochondria and germinal granules at the vegetal pole

aggregate to form approximately four large clusters of germ plasm [103]. One mass of germ plasm is inherited by each of four vegetal-pole blastomeres at the 32-cell stage [104]. As in *C. elegans*, each of these germline blastomeres divides asymmetrically during cleavage stages to form one germline blastomere, which inherits the germ plasm, and one somatic blastomere. The 2–6 cells inheriting the germ plasm in the late blastula are considered the founders of the germline (presumptive PGCs, or pPGCs). After gastrulation the pPGCs divide symmetrically such that germ plasm is inherited by both daughter cells, and the number of PGCs increases. An interesting feature of *Xenopus* germline development is that, in contrast to *Drosophila*, PGCs are not committed to a germ cell fate until they reach the gonad, as revealed by cell transplantation experiments [105]. Thus, even though vegetal pole cytoplasm from 32-cell embryos injected into somatic blastomeres can induce their differentiation into PGCs [106], germ plasm is not sufficient to determine germline fates irreversibly.
Several putative germ plasm components have been identified based on RNA localization to the vegetal pole. These RNAs include *Xcat-2* [107], *Xlsirts* [108], *XVLG1* [109], *Xpat* [110], mtlrRNA [72], *Xcat-3* [111] and *Xlwnt-11* [112]. Two of these RNAs, *Xcat-2* and mtlrRNA, are detected on germinal granules in oocytes, while *Xlsirts* and *Xlwnt-11* are associated with a fibrillar network in the germ plasm, but not with the germinal granules themselves; the precise nature of this fibrillar network is not known [72, 113]. These RNAs all remain enriched in the germ plasm during embryogenesis. In addition *Xpat* RNA is localized to the germ plasm throughout oogenesis and in early cleavage embryos, and is probably also expressed in PGCs from gastrulation until the formation of the dorsal mesentery. However, evidence for a function in germline development exists only for *XVLG1*, a homologue of *Drosophila vasa*. Although it has not been determined if *XVLG1* is present on germinal granules like Vasa, *XVLG1* has been detected in germ plasm in embryos and is expressed in pPGCs in the late gastrula, in PGCs in the tadpole, and in germ cells in the adult ovary and testis [11, 109]. Perturbation of XVLG1 protein in pPGCs appears to block their differentiation into PGCs, as vegetal blastomeres from 32-cell embryos coinjected with anti-XVLG1 antibody and a fluorescent lineage tracer fail to give rise to fluorescent PGCs in tadpoles [67].
Several other germ plasm components represent good candidates for functional germline factors based on their similarity to known genes in *Drosophila*. *Xcat-2* encodes a protein in the CCHC RNA-binding family of zinc finger proteins similar to *Drosophila* Nos in a 58-amino acid domain [107], whereas mtlrRNA is an essential polar-granule component in *Drosophila*, as discussed above [72]. *Xlsirts* are a family of nontranslat-

able interspersed repeat RNAs believed to function structurally, as they are required to anchor *Vg1* RNA at the vegetal cortex [114]. A structural role for the non-translated *Pgc* RNA in *Drosophila* polar granules has also been proposed [115] (see below). However, since *Vg1* is excluded from the germ plasm [113], it remains unclear what role, if any, *Xlsirts* play in the germline. Germ plasm-associated RNAs are transported to the vegetal cortex during oogenesis via the MC. This process occurs early, localizing RNAs such as *Xcat-2*, *Xlsirts*, *Xlwnt-11* and *Xpat* by stage III of oogenesis [68, 108, 110, 116]. Localization of these RNAs occurs in three steps: first, in pre-stage I oocytes RNAs associate with multiple pre-MC structures surrounding the germinal vesicle [117]. Second, in stage I oocytes RNAs are translocated to a single MC lying at the ventral side of the germinal vesicle. RNAs are then sorted within the MC, such that several RNAs show distinct distributions by early stage II. Third, RNAs are translocated to the vegetal cortex within the MC, which is localized at the vegetal pole in stage II. By stage III, RNAs are anchored at the cortex and appear as a disk at the apex of the vegetal pole. The region of the MC that facilitates the transport of these RNAs has been termed the message transport organizer, or METRO [116].

The cytoskeleton also plays a critical role in the early localization of RNAs, as well as the assembly of the MC. Neither microtubules nor actin microfilaments are required for initial targeting of RNAs to the METRO [117], but microtubules are required for MC migration to the vegetal pole, as well as for the aggregation of germ plasm in embryos [102]. *Xenopus* kinesin-like protein 1 (Xklp1) is also required for germ plasm aggregation, confirming the key role of the cytoskeleton in this process [118]. Recently it has been shown that, in addition to polymerized microtubules, the MC in stage II oocytes is rich in spectrin and γ-tubulin [113, 119]. The MC may therefore act as a microtubule organizing center during oogenesis.

A second, later mechanism of RNA localization has been described for somatic factors, such as *Vg1* and *VegT*, that are implicated in mesoderm induction [68, 116, 120, 121]. These RNAs are localized to the vegetal pole but excluded from the MC; indeed, the early pathway may serve to segregate germline components away from somatic factors early in oogenesis. However the two RNA localization pathways are also related, since *Vg1* localization overlaps METRO-localized RNAs at the vegetal pole, suggesting that *Vg1* may use a pathway established earlier by the MC [116]. This idea is consistent with the hypothesis that the MC could serve as a microtubule organizing center, establishing tracks of microtubules that are required for *Vg1* localization later [119]. In this model, the development of the machinery for the late localization pathway would depend on the prior function of the early pathway. Exogenous *Xcat-2* and *Xpat* RNAs injected into stage III oocytes, when the MC already resides at the vegetal pole, can use the *Vg1* pathway to concentrate at the vegetal pole, even though the endogenous transcripts use the METRO pathway [110, 122]. Moreover, sequences in the *Xcat-2* 3′ UTR required for localization via both pathways map to the same region, although they are not identical [122, 123]. Thus, the relationship between the two pathways for vegetal localization remains unclear. Cis-acting sequences both necessary and sufficient for localization to the vegetal cortex have also been mapped for *Xlsirts* and *Xpat* [108, 110], but factors that bind METRO-localized RNAs have not yet been identified.

Mammals

The determination of the germline in mammals differs in several ways from that in *Drosophila*, *C. elegans* and *Xenopus*. First, it does not appear to rely on inherited factors, but rather on positional information in the embryo [124]. Neither germ plasm nor germinal granules have been identified in mammals; electron-dense fibrillogranular 'nuage' material has been seen in mammalian germ cells [2], but its significance is unknown. In addition, in mammals the germline is segregated from the soma much later, during gastrulation instead of early embryogenesis. Precisely when and where PGCs arise in mouse embryos has been studied using clonal analysis [125]. In these experiments, single cells of the pregastrulation epiblast are injected with a fluorescent dextran. Embryos are then cultured for 40 h, and the positions of the injected cell's descendants are determined; PGCs are identified by staining for alkaline phosphatase. These experiments demonstrated that before gastrulation (embryonic day 6, E6) and during early gastrulation (E6.5), PGCs arise from the proximal part of the epiblast. At these stages, cells from which PGCs arise are evenly distributed in a belt encircling the part of the epiblast adjacent to the extraembryonic region. However, at this stage the germline has not yet segregated from the soma, since these cells also give rise to many cells in the extraembryonic mesoderm. Moreover, cell fates are not determined at these stages, as proximal regions of the epiblast grafted onto distal positions can acquire somatic fates instead of germline fates, and vice versa [126]. These findings also indicate that the ability to form germ cells is not restricted to specific cells in the epiblast. Lineage restriction in the germline is believed to occur during the first 16 h of gastrulation, around E7, when PGCs can be identified in the extraembryonic mesoderm, posterior to the primitive streak. At this stage PGCs are first distinguished from other cells by the expression of alkaline phosphatase [127, 128]. Estimates for the size of the found-

ing population of germ cells at this time range from approximately 45 (as determined by clonal analysis and Oct-4 expression (discussed below)) to 125 (based on alkaline phosphatase expression) [125, 128, 129].

Maintenance of the germline

After the initial determination of the germline, germ cells are kept separate from somatic cells throughout development. It is critical that germ cells do not respond to factors promoting somatic differentiation in other embryonic cells, and that they remain totipotent in order to form the reproductive cells for the next generation. One characteristic that distinguishes somatic cells from germline cells in *C. elegans* and *Drosophila* is the ability to produce new mRNAs in early embryogenesis. In both organisms, germ cells begin zygotic transcription later than somatic cells. In *Drosophila* this difference between germline and somatic cells was detected more than 20 years ago, when it was observed that in the blastoderm only somatic nuclei incorporate [³H]uridine triphosphate (UTP) and hybridize to [³H]poly-U [130, 131]. New transcripts can be detected at stages 3–4 of embryogenesis in somatic cells, whereas in germline cells transcripts are not seen until stage 9, during pole cell migration. In *C. elegans* no embryonic mRNAs have been detected in germline blastomeres before the 28-cell stage, whereas somatic blastomeres begin transcription at the 4-cell stage [132]. PGCs in both *C. elegans* and *Drosophila* also lack a specific form of phosphorylated RNA polymerase (RNAP) II that is found in somatic cells in early embryogenesis [133]. The phosphorylated form of RNAP II is not detected in germline blastomeres in *C. elegans* until the 100-cell stage, or in pole cells in *Drosophila* until around stage 7, shortly before the earliest RNAP II-dependent transcript (*vas*) is detected in stage 9 [134]. Moreover, it has recently been demonstrated in *Drosophila* that even the powerful transcriptional activator Gal4-VP16 cannot stimulate transcription before stage 9 when introduced into pole cells, suggesting that early germ cells are refractory to RNAP II-dependent gene expression [135]. While the presence of the phosphorylated form of RNAP II is correlated with the onset of transcription in germ cells, in *Drosophila* at least it is not strictly required, since RNAP II phosphorylation is not altered in *nos* mutants which show premature expression of germline markers [133, 136].

Suppression of zygotic transcription in the germline during embryogenesis in *C. elegans* depends on the *pie-1* gene. In *pie-1* mutants, germline blastomeres adopt somatic cell fates [94], and this phenotype is correlated with both the derepression of transcription in germline blastomeres and the early presence of phos-

phorylated RNAP II in germline blastomeres [132, 133]. *pie-1* encodes a novel protein with two copies of a motif that has been proposed to form zinc-finger domains. The PIE-1 protein is localized to germline blastomeres at the two-cell stage and is found both in the nucleus and in P granules [93, 137]. Ectopic expression of PIE-1 protein in somatic blastomeres causes a significant reduction in the number of new transcripts in those cells, indicating that PIE-1 is both necessary and sufficient for repression of RNAP II transcription. Since PIE-1 suppresses both the production of new mRNAs and phosphorylation of RNAP II, it is likely that these phenomena are linked, suggesting that a general block in RNAP II transcription promotes germline differentiation, possibly by preventing maternally provided transcriptional activators from directing somatic development.

Remarkably, no homologue for PIE-1 has yet been identified in other organisms, but Oct-4 may play a similar role in maintaining the totipotency of the germline in mice. Oct-4 is a member of the POU family of transcription factors, and its RNA is present very early in embryogenesis, at the eight-cell stage, throughout the epiblast. During gastrulation (around E8.5) Oct-4 expression is restricted to PGCs, which continue to express Oct-4 until the onset of differentiation into gametes [129, 138]. Oct-4 is believed to maintain a germline identity by suppressing other differentiation pathways, in that way resembling the function of PIE-1. This idea has been supported by recent studies in which the Oct-4 gene was removed using targeted gene deletion [139]. Normally the inner cell mass (ICM) and its successor, the epiblast, are stem cell populations that give rise to many cell types, including extraembryonic tissues and germ cells. *Oct-4⁻* mice develop to the blastocyst stage but form ICM cells that are not pluripotent. Oct-4 also regulates genes involved in differentiation, such as human chorionic gonadotropin and fibroblast growth factor-4, consistent with a role in establishing and/or maintaining pluripotent cell identities [139, 140]. Since Oct-4 is the earliest germ cell-specific marker identified, and Oct-4 downregulation correlates precisely with loss of potential to form germ cells, it has also been proposed that Oct-4 represents a good candidate for a germline determinant in mammals [141]. However, as *Oct-4* mutant embryos die before the time of germline determination [139], specific disruption of the distal Oct-4 promoter element, which directs Oct-4 expression in PGCs, will be essential to answer this question [129]. Since the germline enhancer element in the Oct-4 promoter is the best germline marker identified so far, identifying elements that bind this enhancer may lead to the isolation of germ cell determinants. Oct-4 may also function similarly in the amphibian *Axolotl*, since AxOct-4 expression is restricted to PGCs during gastrulation [142].

While PIE-1 mediates an early phase of germline regulation in which production of new mRNAs is largely blocked, another mechanism of regulation is required in larvae and adults, when germline transcription is activated, to ensure the proper expression of germline factors as well as to continue to prevent somatic patterns of gene expression. Evidence for germline-specific gene regulation in larvae and adults has come from studying extrachromosomal (EC) arrays. In *C. elegans*, transgenes are heritably transmitted as large linear arrays composed of many tandem repeats of the injected DNA. Although physically present in both germline and soma, most transgenes are not efficiently expressed in the germline, and maternal effect mutations are often rescued only poorly [76]. Moreover it has recently been shown that transgenes for the ubiquitously expressed gene *let-858* are selectively silenced in the germline [143]. While these transgenes are expressed at a low level in the germline initially, within a few generations they are completely silenced, suggesting that different rules govern germline and somatic expression of EC arrays.

Two lines of evidence suggest that differences in transgene expression between the germline and soma may result from differences in chromatin structure in the two cell types. First, coinjecting the *let-858* plasmid with cleaved genomic DNA, forming EC arrays in which the transgene is interspersed with random pieces of genomic DNA, leads to stable expression in the germline [143]. This 'desilencing' depends on the nature of the carrier DNA, since coinjecting with plasmid DNAs does not affect silencing, suggesting that chromatin context is critical for germline expression. Second, *let-858* transgenes are also expressed in the germline in mutants for either *mes-2*, *mes-3*, *mes-4* or *mes-6* [144]. The *mes* (for *maternal-effect sterile*) genes are maternal-effect genes required for proliferation and viability of the germline. All four genes show the same phenotype: embryogenesis appears normal, but in hermaphrodites larval germ cell proliferation is reduced, germ cells degenerate and gametes do not form [145–147]. *Mes-2* and *mes-6* encode homologues of *Enhancer of Zeste* [*E(z)*] and *extra sex combs* (*esc*), respectively [148, 149]. *E(z)* and *esc* are members of the Polycomb group (Pc-G) of proteins in *Drosophila*, which are transcriptional regulators required to maintain homeotic genes in a repressed state, and are believed to act as multimeric complexes that alter local chromatin structure [150]. Since *mes-2* and *mes-6* encode nuclear proteins localized to germline blastomeres by the end of embryogenesis and, like Pc-G genes, depend upon each other for proper localization, they may act similarly to remodel chromatin structure in the germline and thereby repress gene expression. Consistent with this idea, *mes* mutants are sensitive to X-chromosome dosage, suggesting that *mes* genes may

also play a role in the soma early in embryogenesis in dosage compensation, a process which relies on chromatin structure [147]. However, a direct interaction between MES proteins and chromatin has not yet been demonstrated. Intriguingly, although most Pc-G genes are not required for germline development in *Drosophila*, the *mes-2* homologue *E(z)* is required for fertility in females [151].

Context-dependent silencing effects such as position-effect variegation and X-chromosome silencing have been described in flies, yeast, mice and other organisms (reviewed in [152]). Similar mechanisms could be used to maintain germline identity. In *C. elegans*, chromatin morphology appears more compact in germline nuclei than in surrounding somatic cells [143]. It will be interesting to see if this morphological difference is affected in *mes* mutants. In mice, PGCs are undermethylated compared with somatic cells at gastrulation [153], and downregulation of Oct-4 in somatic cells has been associated with changes in chromatin structure and patterns of methylation [154, 155]. Finally, a number of species, such as parasitic nematodes in the *Ascarididae* family, undergo chromatin diminution in somatic cells, resulting in quantitative and qualitative differences in DNA content between germline and soma [156]. It has been hypothesized that DNA elimination functions as an alternate form of transcriptional regulation, in this case preventing germline factors from being expressed in the soma. Repression of transcription may therefore represent an evolutionarily conserved mechanism to distinguish the germline from the soma during development.

Germ cell migration in *Drosophila*

In *Drosophila* the primordial germ cells, known as the pole cells, are initially formed at the posterior of the embryo. During gastrulation the posterior of the embryo invaginates and is displaced anteriorly, carrying the pole cells with it (probably passively) into the interior of the embryo, where they are found in the lumen of the posterior midgut or PMG [35] (fig. 2A). The pole cells migrate first across, and then along, the endodermal cell layer lining the gut towards the overlying mesoderm. Later they separate into two bilateral groups of cells and align with the gonadal mesoderm. Finally, the pole cells and the somatic gonadal precursor cells (SGPs) condense to form the gonad. Although about 40 pole cells are present at the beginning of migration, many are lost during the migration process, and only approximately 20 pole cells populate the gonads [4, 12]. Within the gonad the germ cells begin dividing again when the larva hatches.

The early transendodermal migration is associated with changes in the morphology of both the pole cells and

the gut epithelium. The spherical pole cells become amoeboid and extend pseudopodia during migration, both in vivo and in culture, where they exhibit intrinsic motility [157]. In patterning mutants which affect the differentiation of the gut epithelium, such as *serpent* and *huckebein* (both transform the PMG into the hindgut) and *dorsal* (dorsalizes the PMG), pole cells do not exit the PMG [157–160]. Ultrastructural studies have shown that remodeling of the PMG epithelium occurs during pole cell migration and is required to allow the pole cells to pass through the cell layer [157,

161]; this has also been seen in frog embryos (*Rana pipiens*; [162]). In contrast, mesoderm is not required for the initial transendodermal migration, as pole cells exit the gut in *twist snail* double mutants which completely lack mesoderm [159, 163].

After exiting the gut, pole cells migrate over the surface of the endoderm towards the mesoderm. *Wunen* (*wun*) is required for this step of pole cell migration; in *wun* embryos pole cells move randomly and can be found in tissues they do not usually reach, such as the hindgut primordium [164, 165]. *wun* encodes a 300-amino acid
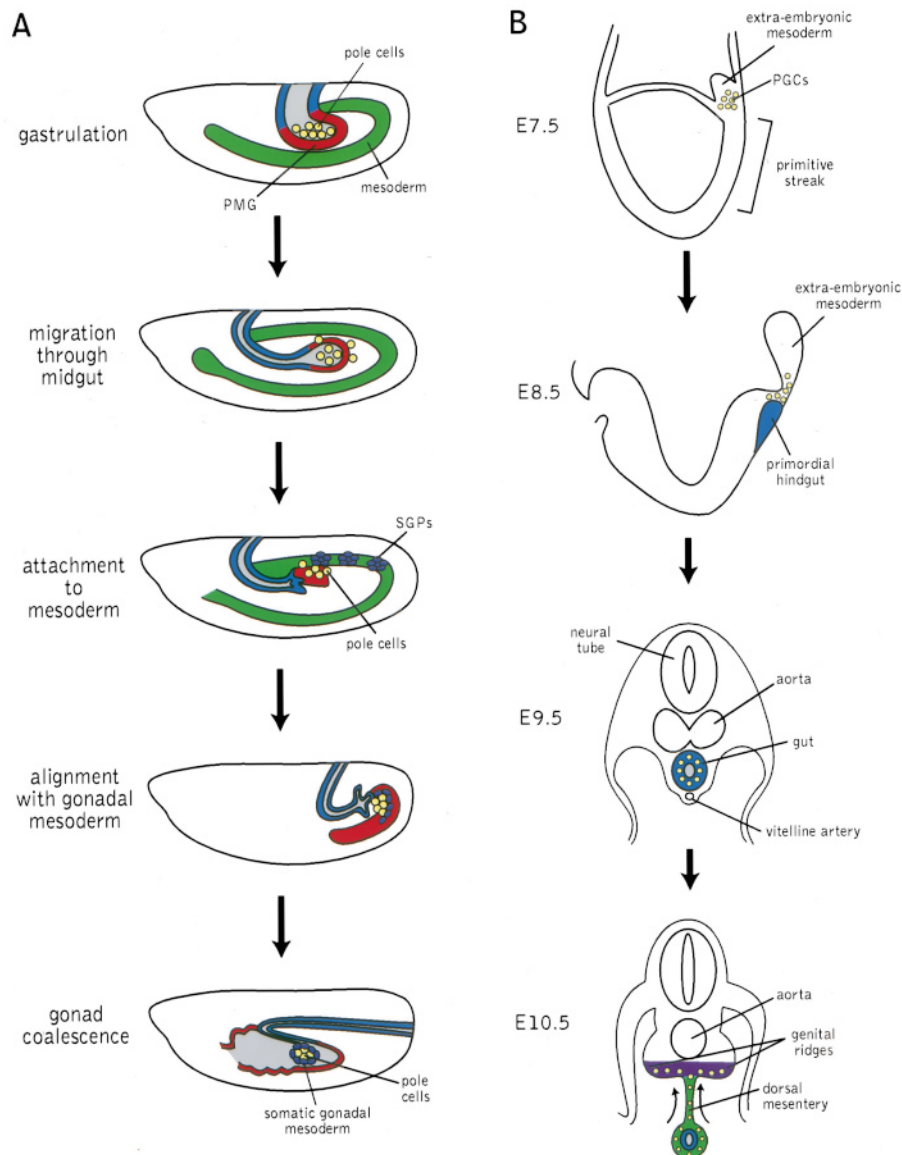

Figure 2. Germ cell migration in *Drosophila* (*A*) and mouse (*B*). *Drosophila* embryos are oriented anterior left and ventral down; mouse embryos are also oriented ventral down. PMG, posterior midgut; SGPs, somatic gonadal precursors; E, embryonic day; PGCs, primordial germ cells. Blue, hindgut; red, posterior midgut; gray, gut lumen; green, mesoderm; purple, somatic gonad primordia; yellow, germ cells. Note the similarities between *Drosophila* and mice: in each case, germ cells are incorporated in the gut and migrate through the mesoderm to the somatic gonadal precursors. (*A*) adapted from [160] and references within; (*B*) adapted from [124].

protein with similarity to the enzyme type-2 phospha-tidic acid phosphatase (PAP2) and six predicted membrane-spanning domains. Two lines of evidence suggest that Wun functions as a specific repellent-guidance factor for germ cells. First, *wun* RNA is expressed in tissues such as the hindgut primordium which usually exclude pole cells, and is expressed on the PMG in a pattern that delimits the region of the gut over which pole cells migrate. Second, expressing *wun* ectopically in the mesoderm blocks PGC migration into the tissue, even though development of the gonadal mesoderm appears normal [165]. It remains unclear how Wun acts to send a repellent signal, and in particular what role, if any, its predicted enzyme activity plays in this process. Similar repulsive mechanisms for guidance have been described in the developing nervous system in vertebrates, where migrating neural crest cells are directed along a specific pathway by repulsive signals from somites [166].

Migration into the mesoderm also requires the correct differentiation of the mesoderm itself, which provides guidance cues to the pole cells. A number of mutations affecting mesoderm development therefore disrupt pole cell migration as well. For example, in *zinc finger home-odomain-1* and *heartless* mutants, most pole cells remain attached to the gut surface and do not associate with mesoderm [160, 167]. A similar phenotype is seen in mutants for *columbus* (*clb*), which is required for pole cell attachment to the mesoderm but not for differentiation of the mesoderm itself, as normal somatic gonads form in *clb* mutants but rarely contain germ cells [160]. Since *clb* is expressed in the gonadal mesoderm and not the pole cells, it represents the best candidate so far for an attractant factor for pole cells [160, 168]. Several transcription factors, such as *abdominalA*, *AbdominalB*, *trithorax*, *trithoraxgleich* and *tinman*, are required for the specification of SGPs [159, 160, 167, 169, 170]. These factors are also required to maintain pole cell association with mesoderm, after the initial attachment has occurred. Finally, mutants for *fear-of-intimacy* and *clift*/*eyes-absent* show morphological changes in the gonadal mesoderm; in these mutants gonad assembly does not occur, even though pole cells and SGPs remain associated [160, 171, 172]. These genes are all required in the soma for pole cell migration, consistent with the idea that somatic tissues provide guidance cues to pole cells [163].

The intrinsic motility of pole cells suggests that they must also contain specific factors involved in movement, in addition to receptors for guidance signals provided by the soma. These germline components are likely maternally expressed, since zygotic transcription in pole cells is repressed until they have reached the primordial gonad, as discussed above. Since screens for mutations affecting migration have looked primarily for zygotically expressed genes, it is perhaps not surprising that so far only two components of the germline, Nos protein and *Polar granule component* (*Pgc*) RNA, have been implicated in pole cell migration. These are both maternal factors which are localized to the posterior pole plasm of the embryo and incorporated into the pole cells as they form.

Nos protein can be detected in the pole cells throughout embryogenesis. *nos* mutant embryos form pole cells; however, since these embryos lack abdomen, the involvement of *nos* in later stages of germ cell development can only be studied under special conditions which allow abdominal development. To investigate the late role of *nos*, *nos*⁻ pole cells were transplanted into a *nos*⁺ embryo [136]. *nos* pole cells in wild-type embryos failed to migrate to the embryonic gonads and hence did not give rise to functional germ cells. Similar results were also seen by examining pole cells in *nos hunchback* (*hb*) double mutants, since the *hb* mutation circumvents the requirement for *nos* in abdomen formation [173]. In both cases, *nos* cells exit the PMG normally, but most of them remain clustered on the outer surface of the gut and do not migrate into the mesoderm. In some embryos a few cells reach the gonadal mesoderm, but these are not incorporated into the gonads. A later requirement for *nos* in maintaining germline stem cells during oogenesis has also been shown [173, 174]. Since *nos* homologues have been identified in several species, it is likely that *nos* function in the germline is conserved (see table 1).

The precise role that *nos* plays in germline development has not been determined. *nos* encodes a zinc-finger RNA-binding protein and represses translation of *hunchback* (*hb*) and *bicoid* (*bcd*) RNAs during embryonic development [175–177]. It may therefore act similarly to regulate the translation of maternally provided transcripts in pole cells, although it must regulate a new set of target RNAs in pole cells, since *hb* repression by *nos* is not required for pole cell migration [136], and *bcd* is not present in pole cells. It has also been reported that premature transcription of germ-cell specific markers occurs in *nos*^BN pole cells, suggesting that *nos* may repress the production of the activator of these late pole cell markers [136] (see below). Recently, a second group observed no premature transcription in *nos* mutant pole cells [178]; however, this group used the *nos*^L7 allele, which, unlike *nos*^BN, retains partial function [51, 174].

The second germline component required after germ cell formation is *Polar granule component* (*Pgc*), an untranslatable RNA which is localized in polar granules and incorporated into pole cells [115]. In embryos with reduced *Pgc* function (produced by transgenic flies expressing antisense *Pgc*), the majority of pole cells are unable to migrate to the gonad and develop into functional germ cells [115]. *Pgc* is therefore essential for the

establishment of the germline, although its exact role is not yet clear. Embryos with less *Pgc* function initially form only slightly fewer pole cells than wild-type embryos, but the pole cells show reduced concentrations of pole plasm components such as *nos* and *gcl* RNAs and Vas protein. A few pole cells do reach the gonads in embryos from females expressing antisense *Pgc*, most likely reflecting a redundancy of *Pgc* function in pole cell migration, or a retention of some *Pgc* activity in the antisense lines.

Germ cell migration and proliferation in vertebrates

In most animals, as in *Drosophila*, the primordial germ cells (PGCs) arise early in development at a site separate from that where the somatic gonads later form, necessitating a complex migration through the embryo to the developing gonad. PGCs divide continuously during migration until they reach the gonad, where they enter either mitotic arrest or meiosis and differentiate into gametes. PGC migration is remarkably similar in such diverse animals as *Drosophila*, *Xenopus*, chick and mouse (reviewed in [179]). Germ cells do not migrate in *C. elegans*, although proliferation still occurs before differentiation of the gonad is complete; the two progenitors of the germline, Z_2 and Z_3, divide continuously throughout larval stages and in the adult to form about 1000 germ cells per gonad [76].

In *Xenopus* the four cells nearest the vegetal pole of the 32-cell stage embryo inherit the germ plasm and ultimately give rise to the germline, which is established by the time of gastrulation. PGCs become incorporated into the developing hindgut during gastrulation, in what is probably a passive process, and then migrate actively through the hindgut mesentery, dorsally and laterally to the genital ridges [179]. During migration the PGCs divide approximately three times so that 20–30 cells populate the genital ridges. In the chick, PGC migration is slightly different, as PGCs travel through the bloodstream before associating with the hindgut mesentery (reviewed in [180]).

In mammals the behavior of PGCs has been studied for over 40 years. Similar patterns of migration and proliferation have been described for mice, rats, humans and others, including marsupials (reviewed in [124]). In mice, where migration has been best characterized, PGCs are first distinguished from other cells just after embryonic day 7 (E7) by the expression of alkaline phosphatase [127]. At this stage they are located in the extraembryonic mesoderm posterior to the primitive streak (fig. 2B). When this region invaginates during gastrulation, PGCs are carried into the embryo and localized to the epithelium of the developing hindgut by E8.5. At E9.5 they begin migration through the dorsal

mesentery to the genital ridges, which are reached around E11.5. Like pole cells in *Drosophila*, mouse PGCs develop pseudopodia and an amoeboid appearance when they enter their migratory phase [181]. Migratory PGCs link up to each other and form extensive networks, both in vivo and in culture, suggesting that aggregation may be an important component of PGC migration; indeed, *Drosophila* PGCs have been shown to interact with each other in a similar way [157]. During migration the number of PGCs increases from a founder population of approximately 100 cells at E7-8 to roughly 25,000 in the genital ridge at E13 [182].

In the mouse most mutations affecting PGC development act in embryos older than E8.5. These mutations typically lead to a reduction in the number of germ cells in the embryo and/or adult, and cause sterility when homozygous. In addition they are usually pleiotropic. The most common phenotype is a failure of PGCs to populate the genital ridges; ectopic PGCs are rarely observed, probably because they undergo apoptosis [183]. Indeed PGCs can survive in culture only if plated on feeder cells [184], consistent with the idea that the proper cellular environment is critical for PGC viability. However, these findings complicate the interpretation of mutations affecting PGC development in the mouse, as it is extremely difficult to distinguish between defects in the survival, proliferation and migration of PGCs.

Two of the best-characterized genes in the mouse are *Dominant white-spotting* (*W*) and *Steel* (*Sl*). *W* encodes the c-kit receptor tyrosine kinase and is expressed on the surface of PGCs (in addition to other cell types). *Sl* encodes the c-kit ligand, often called Steel factor (SF), and is expressed in a gradient along the PGC migratory pathway and in the PGC target tissues, the gonadal ridges [185, 186]. Although studied extensively in cell culture experiments, the precise role that SF/c-kit signaling plays in PGC survival, proliferation and/or motility remains controversial (reviewed in [124, 179, 187]). SF/c-kit signaling inhibits apoptosis of PGCs in culture [183], and recently SF/c-kit interactions have been shown to mediate adhesion of PGCs to somatic cells in culture [188], suggesting that SF/c-kit may promote PGC growth in vivo by promoting adhesion to somatic cells, facilitating signaling between the two cell types.

A third gene product necessary for PGC development, TIAR, has recently been identified [189]. *Tiar* is highly expressed in PGCs and encodes an RNA-recognition motif/ribonucleoprotein-type RNA-binding protein; the same RNA-binding motif is found in Bruno, a translational repressor required for germline development in *Drosophila* [37]. In TIAR mutants, the number of PGCs populating the genital ridge is severely reduced by E11.5, and PGCs are completely absent from the genital ridge by E13.5. Thus, mice lacking TIAR do not form

gametes, most likely due to defects in the survival of migrating PGCs. A similar phenotype is seen in *germ-cell deficient* (*gcd*), a recessive mutation caused by the insertion of a transgene construct [190]. *gcd* also causes a reduction in the number of germ cells as early as E11.5. The *gcd* locus has been mapped to chromosome 11A2-3 but has not been analyzed molecularly [191].

Many different types of growth factors have been shown to affect PGC survival and proliferation in culture (reviewed in [187]), including leukemia inhibitory factor (LIF) [192], basic fibroblast growth factor (bFGF) [193], interleukin-4 (IL-4) [194], pituitary adenylate cyclase-activating polypeptide (PACAP) [195], the Gas 6 ligand for the Sky receptor tyrosine kinase [196] and transforming growth factor (TGF)-β1 [197]. Although several of these, such as IL-4, PACAP and Gas 6/Sky, are expressed at the right time and place to be involved in PGC development, SF remains the only factor known to be required in vivo. Like many migratory cell types PGCs also rely on interactions with the extracellular matrix during migration (reviewed in [198]). Laminin is of particular interest, since in culture it affects both mouse and *Drosophila* PGCs, and in *Drosophila* mutations in laminin A affect pole cell migration to the gonad [157, 199].

In conclusion, there are striking parallels between PGC migration in invertebrates and vertebrates. PGCs are formed away from the gonadal precursors, are carried into the developing gut during gastrulation and migrate actively to the gonadal mesoderm, where they populate the gonads. PGCs acquire a distinctive morphology during migration and are intrinsically motile in culture, but also rely on cues from the surrounding somatic tissues and the target tissues. However, it is not yet clear to what extent the molecular mechanisms underlying these behaviors are conserved. Genetic screens in *Drosophila* have identified several factors involved in signaling from the soma to the germline, whereas in mammals a number of growth factors that may affect PGC migration have been identified in vitro. Perhaps these different approaches will converge and lead to a deeper understanding of the signals involved in germ cell migration.

Postmigratory germ cells

In most species the arrival of PGCs at the gonadal mesoderm marks the end of their proliferative phase and the onset of differentiation into gametes. In *Drosophila* and mice, characteristic changes occur in germ cells when they stop migrating. Germ cells in the gonad lose the amoeboid shape characteristic of migratory cells and gradually lose the capacity to move in vitro [159, 184]. In mice these morphological changes

are associated with changes at the cell surface, such as the loss of several cell surface antigens, and decreased adhesion to fibronectin [124]. Many changes in gene expression also occur in the gonad: in *Drosophila* the majority of pole cell-specific genes are not transcribed until proliferation is reinitiated in the gonads in the final stages of embryogenesis [134], and in mice germ cells acquire sex-specific methylation patterns during gametogenesis [200, 201]. Notably, Oct-4 is downregulated in both oogenesis and spermatogenesis at entry into meiosis, suggesting that in mice Oct-4 downregulation may represent a molecular trigger for commitment to meiosis [141]. Once established in the gonads, germ cells begin to differentiate into either eggs or sperm, depending on the sex of the gonad. Oogenesis and spermatogenesis are complex processes characterized by a period of mitotic proliferation followed by meiosis, and have been reviewed extensively [76, 202–204]. A few of the striking similarities seen in late germline development in a wide range of organisms will be discussed here.

First, mechanisms for regulating the transition from mitotic proliferation into meiosis and progression through meiosis appear to be highly conserved. One of the best examples of evolutionary conservation is seen in the *Deleted in Azoospermia* (*DAZ*) family of genes. *DAZ* was originally identified by a deletion of the Y chromosome in infertile men [205], and autosomal homologues have since been found in humans (*Dazla*) [206–208], mice (*Dazla*) [209], *Xenopus* (*Xenopus DAZ*-like, or *Xdazl*) [210] and *Drosophila* (*boule*) [211]. The human, mouse and fly genes are all expressed predominantly in the testis, required for sperm production, and likely to function similarly, since *DAZ* and *boule* are both required for progression through meiosis. Moreover *Xenopus Xdazl* can rescue meiotic entry in *Drosophila boule* mutants, indicating functional conservation [210]. In addition to a variable number of novel DAZ repeats, these proteins all contain a ribonucleoprotein (RNP)-type RNA binding domain, and *Xdazl* has been shown to bind homopolymeric RNAs in vitro. DAZ function may therefore involve the posttranscriptional regulation of factors required for meiosis. Mouse *Dazla* is also required for female gametes and has been implicated in earlier stages of germ cell development, possibly in PGC proliferation in the fetus. *Xdazl* may also act early in development, since *Xdazl* RNA is localized to the germ plasm in early embryos.

In many organisms germline stem cell divisions are also regulated by somatic cells, although it remains to be seen to what extent the molecular mechanisms underlying somatic signaling are conserved. In *C. elegans* mitotic proliferation in the gonad depends on the somatic distal tip cell (DTC), which inhibits entry into meiosis via the *glp-1* signaling pathway [212]. The *glp-1* signal-

ing pathway is homologous to the *Notch* signaling pathway, which functions in the determination of cell fate in a number of cell types in *C. elegans*, *Drosophila* and vertebrates [213, 214]. In the *Drosophila* ovary, somatic terminal filament (TF) cells may regulate stem cell divisions similarly to the DTC [134], whereas in the testis the TGF-*β* signal transducers *punt* and *schnurri* are required in somatic cyst cells to restrict germ cell proliferation [215]. TGF-*β* has also been shown to suppress proliferation of mouse PGCs in culture [193], suggesting a conserved role for it in regulating germ cell division. Whereas entry into meiosis is also clearly regulated by factors produced by the somatic gonad in mice [216], the molecular nature of these signals has not been determined.

Concluding remarks

Comparative analysis of a number of diverse animals, including both invertebrates and vertebrates, has highlighted several remarkable features of germline development. First, extensive similarities in germ cell development can be found throughout the animal kingdom. For example, perinuclear nuage represents an almost universal feature of germ cells, although its significance still remains unclear. In most animals, germ cells are separated from the soma in embryogenesis. They are internalized during gastrulation and undergo stereotypical migrations from the gut to the somatic gonadal primordia, involving both intrinsic and extrinsic cues. Germ cell differentiation also requires complex mechanisms of gene regulation, involving both transcriptional and translational control. It was proposed 30 years ago that translational regulation in the germline may represent a fundamental means of ensuring the proper expression of germline factors [217]. This idea is now supported by the large number of germline-specific factors that are either localized to the germ plasm as RNAs or that function as translational regulators, and translational regulation appears to represent a conserved mechanism for restricting gene activity in the germline. Germline determination for many animals also relies on the localization of germline factors, although mechanisms used for localization differ. For example, *oskar* may play a unique role in organizing the germ plasm in *Drosophila*; it remains to be seen to what extent mechanisms for localization are species-specific. Similarities in germline development are underscored by the increasing number of factors that have been conserved throughout evolution (see table 1). *vas*, in particular, may represent a universal marker of the germline, as it has been found in a diverse array of organisms, and is localized to germ cells in every case. It is likely that additional germline factors that have not yet been

identified will also be conserved. For a few genes such as *vas*, more than one homologue has been found to exist in some organisms. This finding may reflect a requirement for redundancy in the germline, since factors that ensure the efficiency of germline development would undergo strong selective pressure in evolution. In this regard it is interesting to note that no true 'grandchildless' mutation has been isolated in *Drosophila* to date. It will be of great interest in future studies to determine to what extent conserved genes perform the same functions in different organisms, and whether multiple homologues have diverged in function or merely represent a fail-safe mechanism to ensure the propagation of the species.

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