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Less is more: specification of the germline by transcriptional repression

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In animals, the germline is the only lineage that transmits genetic information to the next generation. Although the founder cells of this lineage are specified differently in invertebrates and vertebrates, recent studies have shown that germline specification in *C. elegans*, *Drosophila* and mouse depends on the global inhibition of mRNA transcription. Different strategies are used in each organism, but remarkably most target the same two processes: transcriptional elongation and chromatin remodeling. This convergence suggests that a repressed genome is essential to preserve the unique developmental potential of the germline.

Introduction

A central goal of developmental biology is to understand how cells adopt specific fates during development. In somatic lineages, cell fate specification often depends on the activation of complex transcriptional programs by tissue-specific transcription factors. For example, specification of skeletal muscle depends on the MyoD family of basic helix-loop-helix transcription factors. Expression of MyoD or of other members of the family is sufficient to activate the transcription of muscle-specific genes (Berkes and Tapscott, 2005). Germ cell development also involves a complex differentiation program to generate the gametes, but in addition germ cells must maintain the potential to form all cell types at fertilization. In this review, we summarize our understanding of the transcriptional mechanisms that contribute to the specification of the germline in *Caenorhabditis elegans*, *Drosophila melanogaster* and mice (Fig. 1). Surprisingly, the emerging theme is one of transcriptional repression, not activation.

Germ plasm specifies germ cell fate

The search for molecules that determine germ cell fate began in 1974 after Mahowald and colleagues showed that cytoplasm taken from the posterior of a *Drosophila* embryo (germ or pole plasm) was sufficient to induce ectopic germ cells, when injected into a host embryo (Illemensee and Mahowald, 1974). Mahowald's experiments defined the criteria for a germline determinant: it should be present in the germ plasm, it should be required for germ cell formation, and it should be sufficient to induce germ cells when expressed elsewhere in the embryo. Genetic screens identified several factors required for germ cell development that localize to the posterior pole of the *Drosophila* embryo (Santos and Lehmann, 2004), but only one factor satisfied all three criteria: Oskar. Oskar is a *Diptera*-specific protein, that, when expressed at the anterior of the *Drosophila* embryo, is sufficient to assemble germ plasm and induce germ cell fate (Ephrussi and Lehmann, 1992). The biochemical function of Oskar remains enigmatic, but it appears to be linked to endocytosis and to actin remodeling, to create a platform that recruits and anchors factors essential for germ cell development (Breitwieser et al., 1996; Tanaka and Nakamura, 2008; Vanzo et al., 2007). These factors, including several RNA-binding proteins and mRNAs, assemble in large ribonucleoprotein (RNP) complexes, called polar granules. Similar cytoplasmic complexes (generically called germ granules) have been observed in the germ lineages of many animals (Eddy, 1975; Extavour and Akam, 2003). In *C. elegans*, segregation of the germ granules (called P granules) during early cleavages correlates with segregation of the germline (Strome and Wood, 1982). The current hypothesis is that germ granule components collectively specify germ cell fate, as none (besides Oskar) is sufficient to induce germ cells on its own. Surprisingly, none of the germ plasm components with a demonstrated role in germ cell formation resembles known transcriptional activators, challenging the idea of a germline equivalent to MyoD in muscle.

Transcriptional quiescence in early germ cells of *Drosophila* and *C. elegans*

A first hint that specification of the germline might involve transcriptional repression rather than activation came from the work of Marko Zalokar. Zalokar found that incubation of early *Drosophila* embryos with the mRNA precursor [H^3]-uridine resulted in the labeling of somatic nuclei but not of germline nuclei (Zalokar, 1976). Germ (pole) cells only became labeled late in gastrulation, just before initiating their migration out of the midgut to the somatic gonad. Hybridization experiments to detect nuclear poly(A)$^+$ mRNAs (Kobayashi et al., 1988; Lamb and Laird, 1976) confirmed that the activation of mRNA transcription is delayed in the germ lineage. Years later, studies in *C. elegans* reached the same conclusion, by using in situ hybridization to reveal the presence of zygotic mRNAs in somatic blastomeres but not in germline blastomeres (Seydoux and Fire, 1994; Seydoux et al., 1996). As newly transcribed ribosomal RNAs are present in both cell types, it is mRNA transcription that appears to be specifically blocked (Seydoux and Dunn, 1997).

mRNA transcription is tightly coupled to the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII) (Fig. 2). The CTD comprises tens of copies of the heptapeptide motif YSPTSPS (42 in *C. elegans*, 42 in *Drosophila* and 52 in mammals), which become phosphorylated at Ser5 during transcriptional initiation and at Ser2 during elongation (Corden, 2007; Peterlin and Price, 2006; Saunders et al., 2006). In *Drosophila* and *C. elegans* embryos, phosphorylated CTD Ser5 and Ser2 (hereafter pSer5 and pSer2) appear in somatic nuclei at the onset of zygotic transcription. By contrast, only low levels of pSer5, and no pSer2, are detected in early germ cell nuclei, suggesting that a block occurs at a step of initiation and elongation (Seydoux and Dunn, 1997).

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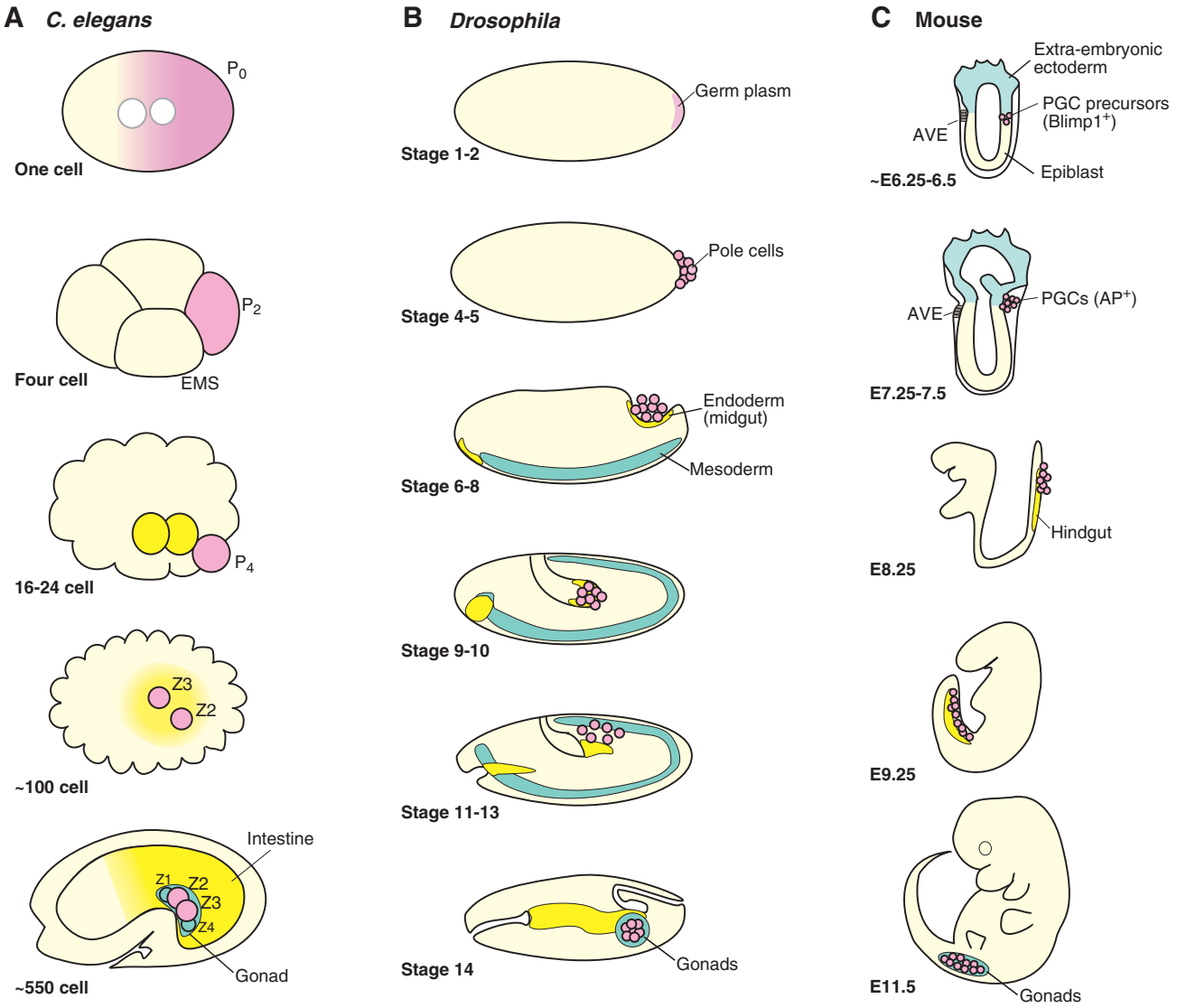

Fig. 1. Early germ cell development in *Drosophila*, *C. elegans* and mice. (A,B) Anterior is towards the left. (**A**) In *C. elegans*, the germ plasm (pink) becomes asymmetric in the zygote and segregates specifically to the germline blastomeres P_1-P_4. Z2 and Z3, the daughters of P_4, become the primordial germ cells (PGCs), and move inside the embryo in close association with intestinal cells (yellow). Later they are joined by Z1 and Z4, the founder cells of the somatic gonad. (**B**) *Drosophila*: germ plasm (pink) is assembled during oogenesis and preformed in the posterior pole region of eggs. Pole cells incorporate germ plasm, and are carried inside the embryo during germ band extension, pass through the midgut epithelia (yellow) into the hemocoel, migrate toward mesoderm (green), and then coalesce with somatic gonadal cells to form the embryonic gonads. (**C**) The anterior-posterior axis of mouse embryos is established by the anterior visceral endoderm (AVE). During embryonic day (E) 6.25-6.5, extra-embryonic signals promote four to eight proximal epiblast cells to activate Blimp1 expression (pink). These cells migrate to an extra-embryonic location and by E7.25, have proliferated to form ~40 alkaline phosphatase (AP)-positive PGCs that are present at the root of the allantois. These PGCs migrate back into the embryo in association with the hindgut (yellow) to eventually colonize the genital ridges (somatic gonad).

What prevents transcription in early germ cells? Maternal expression of Gal4-VP16, a potent transcriptional activator, is not sufficient to activate a synthetic Gal4 target in *Drosophila* pole cells. By contrast, the ectopic assembly of germ plasm in somatic cells at the anterior pole of the early *Drosophila* embryo is sufficient to inhibit transcription induced there by the transcription factor Bicoid (Van Doren et al., 1998). The lack of transcription in pole cells, therefore, is not due to a lack of transcription factors, but instead appears to be an active process, in which at least one germ plasm component inhibits transcription, perhaps by targeting a core factor of the transcriptional machinery. The discovery of novel transcriptional repressors in the germ plasm of *C. elegans* and *Drosophila* has confirmed this prediction, and has revealed new mechanisms for inhibition of RNAPII. In the following, we discuss several of these repressors in more detail. An overview of the repressors is provided in Table 1, and their actions are illustrated in Fig. 2.

C. elegans PIE-1 inhibits transcriptional elongation by inhibiting P-TEFb

pie-1 (*pharynx and intestine in excess*) was first identified in a genetic screen for maternal-effect mutations that mispattern *C. elegans* embryos (Mello et al., 1992). PIE-1 is a maternal protein that segregates with the germ granules in the early embryo and accumulates in the nuclei of the germline blastomeres P_2, P_3 and P_4

(Mello et al., 1996). In *pie-1* mutants, the P_2 lineage is transformed to resemble its sister lineage, EMS. This transformation depends on SKN-1 (skinhead), a maternally loaded transcription factor that is present in the EMS and P_2 blastomeres. In wild-type embryos, SKN-1 activates the transcription of EMS-specific genes in EMS, but not in P_2 because P_2 contains germ plasm and is not transcriptionally active. In *pie-1* mutants, P_2 becomes transcriptionally active and contains high levels of both pSer2 and pSer5. As a result, SKN-1 is free to activate EMS-specific genes, transforming the P_2 lineage into the EMS lineage (Mello et al., 1992; Seydoux et al., 1996; Seydoux and Dunn, 1997; Tenenhaus et al., 2001). Thus, in *C. elegans*, the establishment of the germ lineage depends on the inhibition of mRNA transcription by PIE-1 to prevent SKN-1, and possibly other transcription factors, from inducing somatic programs of differentiation.

The ectopic expression of PIE-1 in *C. elegans* embryos or the binding of PIE-1 to a promoter in human cells is sufficient to repress transcription, suggesting that PIE-1 functions directly as a general transcriptional repressor (Seydoux et al., 1996; Batchelder et al., 1999). The repressor activity of PIE-1 has been mapped in human cells to a domain that contains the sequence YAPMAPT (Batchelder et al., 1999). This sequence resembles a non-phosphorylatable version of the YSPTSPS motif in the RNAPII CTD. Studies in the Peterlin laboratory have shown that tandem copies of non-phosphorylatable CTD repeats (serines replaced by alanines) interfere with transcription by binding to Cyclin T (Zhang et al., 2003). Cyclin T is the CTD-binding subunit of P-TEFb (positive transcription elongation factor b), which phosphorylates CTD Ser2 during transcriptional elongation (Peterlin and Price, 2006; Saunders et al., 2006). Remarkably PIE-1, which only has a single YAPMAPT motif, binds to Cyclin T in vitro and interferes with the stimulation of transcription by P-TEFb in mammalian cells (Zhang et al., 2003). A PIE-1 mutant that lacks the YAPMAPT motif does not bind to Cyclin T and does not interfere with P-TEFb (Zhang et al., 2003). The conclusion from these heterologous cell culture assays is that PIE-1 inhibits transcriptional elongation directly by binding to Cyclin T and by diverting the P-TEFb complex away from the CTD.

Structure/function studies in *C. elegans* embryos have confirmed these findings and added an unexpected twist (Ghosh and Seydoux, 2008). When expressed in *pie-1* mutant embryos, a PIE-1 transgene that lacks the YAPMAPT is unable to suppress pSer2 in germline blastomeres, as expected. Surprisingly, however, the YAPMAPT mutant is able to suppress pSer5 as efficiently as wild-type PIE-1. Even with the abnormally high levels of pSer2, production of zygotic transcripts remains repressed and germ cells are specified normally. Additional sequences around the YAPMAPT need to be deleted to eliminate all transcriptional inhibition by PIE-1 and to cause loss of germ cells (Ghosh and Seydoux, 2008). These unexpected findings suggest that PIE-1 regulates pSer2 and pSer5 independently, using P-TEFb-dependent and P-TEFb-independent mechanisms. Although both mechanisms are used during wild-type development, inhibition of CTD Ser5 phosphorylation is sufficient to inhibit transcription and to specify germ cell fate (Ghosh and Seydoux, 2008). Remarkably, recent studies have shown that P-TEFb is also targeted for suppression in *Drosophila* PGCs.

Drosophila Pgc also blocks mRNA transcription by inhibiting P-TEFb

polar granule component (*pgc*) was originally identified as an RNA component of the *Drosophila* germ plasm (Nakamura et al., 1996). A reduction in *pgc* mRNA levels, either by expressing an antisense *pgc* transcript (Nakamura et al., 1996) or through a loss-of-function mutation in *pgc* (Hanyu-Nakamura et al., 2008; Martinho et al., 2004), does not interfere with pole cell formation but causes pole cells to degenerate during mid-embryogenesis. Pole cells that lack *pgc* are positive for pSer2 and express several transcripts that are normally expressed only in neighboring somatic cells (Deshpande et al., 2004; Hanyu-Nakamura et al., 2008; Martinho et al., 2004).

The initial characterization of the *pgc* locus suggested that *pgc* might act as a non-coding RNA (Nakamura et al., 1996). Subsequent analyses, in which the *pgc* locus was compared in 12 *Drosophila* species, revealed the presence of a small open-reading frame coding for a conserved 71 amino acid protein (Hanyu-Nakamura et al., 2008; Timinszky et al., 2008). Pgc protein is expressed transiently in early pole cells (stages 4-5) at the time when pole cell nuclei begin to loose pSer2 signals. The Pgc expression pattern is precisely complementary to the pSer2 pattern in pole cells. Furthermore, ectopic expression of Pgc in the anterior pole of the *Drosophila* embryo is sufficient to interfere with pSer2 accumulation and with Bicoid-dependent gene expression in somatic cells, suggesting that Pgc is the germ plasm component that represses mRNA transcription (Hanyu-Nakamura et al., 2008; Timinszky et al., 2008).

Pgc does not share any sequence similarity with PIE-1, yet, remarkably, biochemical experiments have shown that Pgc also blocks transcription by interfering with P-TEFb. Pgc exists in a complex with P-TEFb in *Drosophila* embryonic extracts, and interacts with P-TEFb in vitro (Hanyu-Nakamura et al., 2008). Overexpression of P-TEFb in pole cells phenocopies *pgc* mutants, and stimulates CTD Ser2 phosphorylation most efficiently in embryos with low levels of Pgc, suggesting that an antagonistic interaction exists between Pgc and P-TEFb. Pgc does not inhibit P-TEFb activity directly in vitro, but, when expressed in salivary glands, Pgc interferes with the localization of P-TEFb to polytene chromosomes. Chromatin immunoprecipitation (ChIP) experiments in heat-shocked *Drosophila* S2 cells have confirmed that Pgc interferes with the recruitment of P-TEFb to the heat shock-induced Hsp genes (Hanyu-Nakamura et al., 2008).

The exact mechanism by which Pgc interferes with P-TEFb recruitment remains unknown. Unlike PIE-1, Pgc does not contain a CTD-like sequence and does not bind to P-TEFb by means of Cyclin T. Instead, Pgc binds directly to Cdk9, the kinase subunit of P-TEFb that is responsible for CTD Ser2 phosphorylation. Pgc and PIE-1, therefore, appear to have evolved independent mechanisms to inhibit P-TEFb. Whether Pgc also inhibits the phosphorylation of Ser5 is not known. Specification of germ cell fate by maternally inherited germ plasm is thought to have arisen independently several times during evolution (Extavour and Akam, 2003). The fact that Pgc and PIE-1 perform similar functions, yet are structurally unrelated, is consistent with this view, and suggests that the inhibition of elongation may be a versatile mechanism by which to regulate mRNA transcription across the genome.

Interestingly, genome-wide mapping studies of RNAPII in yeast, human and *Drosophila* cells have shown that many ‘silent’ genes maintain high levels of RNAPII at their 5′ ends, suggesting that the stalling of RNAPII is a common mode of repression (Guenther et al., 2007; Muse et al., 2007; Kim et al., 2005; Radonjic et al., 2005; Zeitlinger et al., 2007). In *Drosophila* embryos, the genes with stalled RNAPII are often those that are induced rapidly at later stages of development (Muse et al., 2007; Zeitlinger et al., 2007). RNAPII stalling may therefore be an efficient method by which to silence genes transiently without loss of transcriptional competence

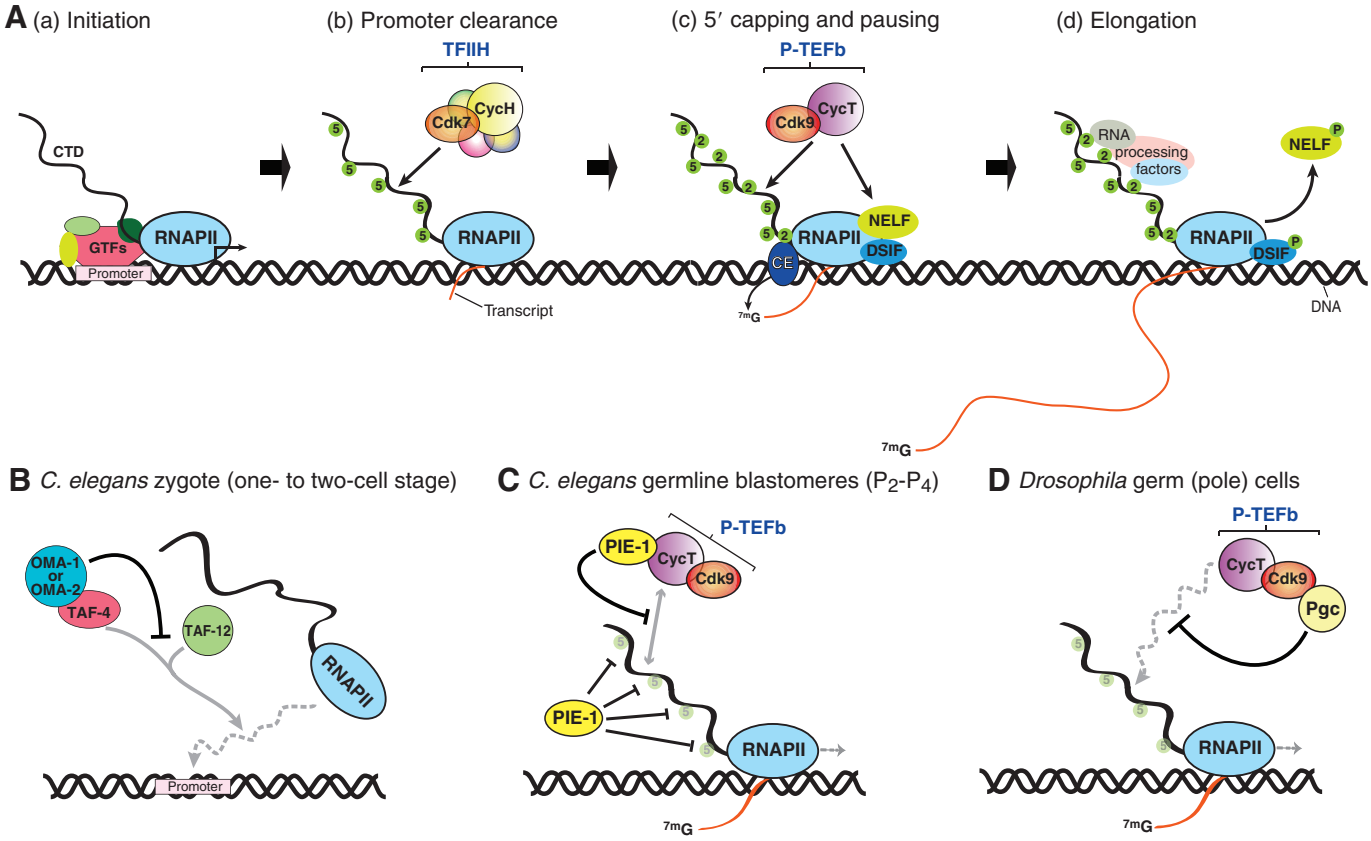

Fig. 2. mRNA transcription and its repression during germ cell specification. (**A**) Steps of RNA transcription. (a) Initiation: the pre-initiation complex [consisting of RNAPII (blue) and the general transcriptional factors (GTFs, pink)] assembles at the promoter. (b) Promoter clearance: Cdk7 (orange) in the TFIIH complex phosphorylates Ser5 in the CTD repeats (green circles), allowing the polymerase to clear the promoter and recruit capping enzymes. (c) 5′ capping and pausing: shortly after initiation, RNAPII is paused by the action of negative factors [DISF (DRB sensitivity-inducing factor) and NELF (negative elongation factor)]. P-TEFb (positive transcription elongation factor b) phosphorylates CTD Ser2 (green circles), DISF and NELF, promoting the dissociation of NELF and the conversion of DSIF into a positive elongation factor, leading to productive elongation (d). CTD Ser2 phosphorylation also promotes recruitment of mRNA-processing enzymes. (B-D) Repression of mRNA transcription by germline proteins. (**B**) In one- to two-cell stage *C. elegans* embryos, maternally loaded OMA-1 and OMA-2 compete with TAF-12 for binding to the GTF TAF-4, keeping it sequestered in the cytoplasm. (**C**) In the P_2-P_4 blastomeres, PIE-1 interacts with the Cyclin T subunit of P-TEFb, blocking its interaction with the CTD and Ser2 phosphorylation. PIE-1 also inhibits Ser5 phosphorylation by an unknown mechanism. (**D**) In *Drosophila* pole cells, Pgc binds to the Cdk9 subunit of P-TEFb, and prevents P-TEFb recruitment to chromatin (broken gray arrow). The mechanism that blocks Ser5 phosphorylation is not known.

('reversible repression'). Consistent with this view is the fact that, upon the loss of PIE-1, transcription in *C. elegans* blastomeres is re-activated within one cell division (Seydoux et al., 1996).

Other germ plasm proteins that repress transcription in early germ cells

In *C. elegans* embryos, mRNA transcription in somatic blastomeres starts at the three- to four-cell stage. Before that, the zygote (P_0) and the first two blastomeres (including the germline blastomere, P_1) are completely negative for both pSer2 and pSer5 (Seydoux and Dunn, 1997). A recent study has now shown that two proteins related to PIE-1 (OMA-1 and OMA-2; oocyte maturation defective) are responsible for this early repression mode (Guven-Ozkan et al., 2008). OMA-1 and OMA-2 are nearly identical proteins that, like PIE-1, are inherited maternally and associate with P granules (Detwiler et al., 2001). Unlike PIE-1, however, OMA-1 and OMA-2 are present at high levels only in the zygote and are rapidly degraded during the two-cell stage. Remarkably, Guven-Ozkan et al. (Guven-Ozkan et al., 2008) found that OMA-1 and OMA-2 bind to TAF-4, a TBP (TATA-binding protein)-associated factor that is essential for transcriptional initiation. OMA-1 and OMA-2 bind TAF-4 through a domain that is related to the histone-fold domain of TAF-12, a TAF-4-binding protein required for TAF-4 nuclear localization. In one- and two-cell stage embryos, OMA-1 and OMA-2 compete with TAF-12 for TAF-4 binding, keeping TAF-4 in the cytoplasm. The stabilization of OMA-1 and OMA-2 beyond the two-cell stage is sufficient to block pSer2 accumulation in somatic blastomeres, demonstrating that OMA-1/2 are potent inhibitors of transcription (Guven-Ozkan et al., 2008).

Like PIE-1, OMA-1 and OMA-2 belong to a class of RNA-binding proteins characterized by two CCCH finger motifs. In addition to repressing transcription, OMA-1 and OMA-2 have a separate role in oocyte maturation, which may involve the post-transcriptional regulation of oocyte mRNAs (Detwiler et al., 2001). Interestingly, PIE-1 also has an independent second function that is required to stimulate the translation of the P granule-associated mRNA *nanos* homolog *nos-2* (see below) (Tenenhaus et al., 2001). How the RNA-regulating functions of OMA-1, OMA-2 and PIE-1 intersect with their transcriptional functions is not yet known.

In *Drosophila*, germ plasm components other than Pgc have been implicated in transcriptional silencing, but whether they act directly remains under question. For example, Germ cell-less (Gcl) is a nuclear pore-associated protein that is implicated in the earliest stages of transcriptional repression before the cellularization of the pole cells occurs (pole bud stage) (Jongens et al., 1994; Leatherman et al., 2002). However, in these early stages, pSer2 is detectable in interphase pole bud nuclei even in wild-type embryos (Hanyu-Nakamura et al., 2008). At later stages, pSer2 is repressed in the few pole cells that form in *gcl* mutants, indicating that Gcl is not essential to suppress pSer2.

Similarly, *nanos* mutants have been reported to show modest pSer2 accumulation in stage 4 *Drosophila* embryos (Deshpande et al., 2005), although another study has reported that pSer2 is still not fully repressed at the beginning of stage 4 (nuclear cycle 10) even in wild-type embryos (Hanyu-Nakamura et al., 2008). Nanos, a cytoplasmic translational repressor that is essential for several conserved aspects of PGC development, is dispensable for the inhibition of Bicoid-dependent transcription by ectopic germ plasm in *Drosophila* (Van Doren et al., 1998), and is not required for PIE-1-dependent inhibition of pSer2 in *C. elegans* (Schaner et al., 2003). In *Drosophila*, *C. elegans* and mice, the *nanos* gene family is required, directly or indirectly, for the survival, cell cycle arrest, migration and chromatin remodeling (see below) of PGCs (Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Kobayashi et al., 1996; Schaner et al., 2003; Subramaniam and Seydoux, 1999; Tsuda et al., 2003). Its targets in *Drosophila* pole cells include maternal *Cyclin B* mRNA (the repression of which promotes G2 arrest) (Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Kadyrova et al., 2007) and the *head involution defective* mRNA (the repression of which protects pole cells from apoptosis) (Hayashi et al., 2004; Sato et al., 2007). If Nanos also contributes to transcriptional repression in germ cells, it probably does so indirectly (e.g. by inhibiting the translation of a transcriptional activator).

Chromatin-based silencing follows Pgc and PIE-1

The post-translational modification of histones, such as methylation (me), acetylation (ac), ubiquitinylation and phosphorylation, can dramatically affect the transcriptional competence of chromatin. According to the histone code hypothesis, specific histone modifications specify the transcriptional competence of chromatin (Berger, 2007; Jenuwein and Allis, 2001). For example, methylation of histone H3 at lysine 4 (H3meK4) is generally correlated with transcriptionally active chromatin, whereas methylation of H3 at lysine 9 (H3meK9) correlates with inactive chromatin, such as heterochromatin.

In *Drosophila*, pole cell nuclei lack H3meK4 from the time of their formation and show a different pattern of H3meK9 compared with somatic cells (Rudolph et al., 2007; Schaner et al., 2003). Several lines of evidence suggest that this unique chromatin status is not merely a secondary consequence of the lack of RNAPII activity. First, in *pgc* mutants, transcriptionally derepressed pole cells acquire H3meK4, but maintain the H3meK9 pattern (Martinho et al., 2004). Second, the loss of Su(var)3-3, the *Drosophila* ortholog of the H3 K4 demethylase LSD1, causes H3meK4 to appear in early pole cells, suggesting that the absence of H3meK4 depends on its active removal (Rudolph et al., 2007). Whether *Su(var)3-3* mutant pole cells are transcriptionally active remains unknown. Third, when Pgc protein disappears during stages 6-7, pSer2 is upregulated but only low levels of zygotic transcripts (e.g. *zen*) are detected until pole cells become migratory at stage 9 (Martinho et al., 2004; Van Doren et al., 1998; Zalokar, 1976). Removal of Osa, a component of the Swi/Snf chromatin-remodeling complex, causes the robust accumulation of zygotic *zen* transcripts in pole cells as soon as Pgc becomes undetectable (Martinho et al., 2004). These observations suggest that, in *Drosophila*, chromatin-based mechanisms of transcriptional repression are put in place from the time of pole cell formation, and become essential for keeping transcription repressed after Pgc runs out.

In *C. elegans*, the chromatin of germline blastomeres is indistinguishable from that of somatic blastomeres during the period of OMA-1-, OMA-2- and PIE-1-dependent repression, but changes dramatically when PIE-1 disappears from the germ lineage (Schaner et al., 2003). When the last germline blastomere P_4 divides into the PGCs Z2 and Z3, PIE-1 is degraded, the histone linker H1.1 (*his-24*) and the 'active' chromatin modifications H3meK4 and H4acK8 disappear, and Z2 and Z3 take on a unique condensed chromatin appearance (Jedrusik and Schulze, 2007; Schaner et al., 2003) (H. Furuhashi and W. Kelly, personal communication). During these events, Z2 and Z3 become transiently positive for pSer2, probably owing to the abrupt loss of PIE-1, but by mid-embryogenesis, pSer2 is once again strikingly reduced (H. Furuhashi and W. Kelly, personal communication). Analyses of centrosome number and DNA content have revealed that Z2 and Z3 are arrested in G2, or in early prophase, throughout embryogenesis (Fukuyama et al., 2006). Interestingly, *Drosophila* pole cells exhibit a similar cell cycle arrest (Asaoka-Taguchi et al., 1999; Deshpande et al., 1999).

After *C. elegans* larvae hatch and begin feeding, Z2 and Z3 resume cell division and become positive again for pSer2, H3meK4 and H4acK8 on all chromosomes, except for the X chromosome. The X chromosome remains transcriptionally silent throughout germline development, except for a brief period during late oogenesis (Schaner and Kelly, 2006). The silencing of the *C. elegans* X chromosome is so complete that genes expressed in the germline are rarely found on the X chromosome (Reinke, 2006). In *C. elegans*, X chromosome silencing depends on two classes of chromatin proteins: (1) the Polycomb-like complex MES-2/MES-3/MES-6 (maternal effect sterile), which concentrate methylated H3K27 (a repressive mark) on the X chromosome (Bender et al., 2004); and (2) the autosome-associated proteins MES-4 and MRG-1 (mortality factor-related gene) (Bender et al., 2006; Takasaki et al., 2007). MES-4 is a SET domain protein that methylates H3K36 on autosomes (Bender et al., 2006) and MRG-1 is a chromodomain protein of unknown biochemical function (Takasaki et al., 2007). Although specific for autosomes, a microarray analysis of *mes-4* mutants has confirmed that the primary defect caused by loss of MES-4 in adult *C. elegans* gonads is the ectopic activation of X-linked genes in germ cells (Bender et al., 2006). Interestingly, although MES-4 and its H3K36me mark normally appear in a banded pattern on autosomes, in the absence of the MES-2/MES-3/MES-6 complex, this pattern becomes more uniform along the autosomes and spreads to the X chromosomes, revealing that the MES-2/MES-3/MES-6 system regulates the pattern of H3K36me on autosomes, as well as on the X (Bender et al., 2006). Loss of MRG-1 or any one of the MES proteins leads to dramatic germ cell loss in larvae (Fujita et al., 2002; Garvin et al., 1998), indicating that transcriptional repression remains essential in the germline, even long after the inhibition of RNAPII has been lifted.

Transcriptional repression in mice

At first glance, germline formation appears to occur quite differently in mice when compared with *C. elegans* and *Drosophila*. Mouse oocytes do not have germ plasm and all cells in the epiblast appear able initially to contribute to the germline (Tam and Zhou, 1996).

Table 1. Proteins implicated in transcriptional repression during germ cell specification

Species	Protein	Motifs	Function	Roles in germline development	References
C. elegans	PIE-1	CCCH: RNA binding, YAPMAPT: Cyclin T binding	Interferes with CTD Ser2 and Ser5 phosphorylation partly by competing with the CTD for binding to P-TEFb	Specification of germline blastomere P_2, translation of nos-2 in P_4	Mello et al., 1996; Seydoux et al., 1996; Seydoux and Dunn, 1997; Batchelder et al., 1999; Zhang et al., 2003; Ghosh and Seydoux, 2008
	OMA-1/2	CCCH: RNA binding, TAF-4 binding domain	Interferes with transcriptional initiation by keeping TAF-4 in cytoplasm	Transcriptional repression in P_0 and P_1 (PGC precursor), oocyte maturation	Detwiler et al., 2001; Guven-Ozkan et al., 2008
	MES-2/3/6	Polycomb-like complex	Methylation of H3K27 concentrated on the X chromosomes	X-chromosome silencing, germ cell viability (postembryonic)	Bender et al., 2004; Bender et al., 2006
	MES-4	SET domain	Methylation of H3K36 on autosomes	X-chromosome silencing, germ cell viability (postembryonic)	Bender et al., 2006
	MRG-1	Chromodomain (might bind to methylated lysine residues)	Unknown	X-chromosome silencing, germ cell viability (postembryonic)	Takasaki et al., 2007
Drosophila	Pgc	Unknown (binds to the Cdk9 subunit of P-TEFb)	Interferes with CTD Ser2 phosphorylation by sequestering P-TEFb away from chromatin	Transcriptional repression in early pole cells, pole cell maintenance during embryogenesis	Nakamura et al., 2006; Deshpande et al., 2004; Martinho et al., 2004; Hanyu-Nakamura et al., 2008
	Gcl	Unknown	Unknown (present in the nuclear pore)	Pole cell formation	Robertson et al., 1999; Leatherman et al., 2003
	Nos	CCHC: RNA binding	Translational repressor	Repression of *Cyclin B* and *hid* mRNA translation in pole cells to prevent mitosis and apoptosis, respectively, prevents *skl* activation in pole cells (may be indirect)	Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Hayashi et al., 2004; Sato et al., 2007
	Osa	ARID (AT-rich interaction domain) DNA binding	Component of the Swi/Snf chromatin remodeling complex	Repression of zygotic zen transcription after Pgc runs out from pole cells.	Martinho et al., 2004
	Su(var)3-3	Amine oxidase	Removal of methyl groups from mono- and dimethylated K4 in Histone H3	Suppression of H3K4me in early pole cells.	Rudolph et al., 2007
Mouse	Blimp1/ Prdm1	PR domain	Sequence-specific recruitment of Groucho-type co-repressors and chromatin modifiers (as shown in somatic cells)	Transcriptional repression of virtually all downregulated genes in PGCs, activates 50% of upregulated genes	Ohinata et al., 2005; Kurimoto et al., 2008; Kallies and Nutt, 2007
	Prdm14	PR domain	Unknown	Repression of GLP (H3K9 methylase), activation of Sox2	Yamaji et al., 2008

During gastrulation, BMP (bone-morphogenetic protein) signals from extra-embryonic tissues induce proximal cells in the epiblast to commit to the germline fate (Lawson et al., 1999; Ying et al., 2001). Definitive PGCs appear around embryonic day (E) 7.25 as a small cluster of ~40 alkaline phosphatase-positive cells posterior to the primitive streak. The PGCs are initially in an extra-embryonic location and migrate back into the embryo to reach the somatic gonads (genital ridges) by ~E10.5 (Fig. 1). Despite the different modes of specification in mice, flies and worms, recent analyses indicate that PGC specification in the mouse also depends on transcriptional repression.

To identify molecules responsible for PGC specification, the labs of Azim Surani and Mitinori Saitou developed protocols to profile transcripts in individual PGCs isolated directly from mouse embryos (Ohinata et al., 2005; Saitou et al., 2002; Surani et al., 2004; Yabuta et al., 2006). These pioneering experiments led to the identification of scores of transcripts that are upregulated or downregulated during PGC specification (Kurimoto et al., 2008). Among the upregulated group are genes required for pluripotency (such as *Sox2* and *Nanog*) and germ cell development (e.g. *Dnd1* and *Nanos3*). The downregulated group includes genes associated with mesodermal development (such as *Hoxb1*), cell cycle regulation and DNA

methylation. The categories of genes that are downregulated are consistent with the fact that: (1) the somatic neighbors of PGCs follow a mesodermal developmental program that must be blocked in emerging PGCs (Saitou et al., 2002); (2) PGCs arrest in the G2 phase of the cell cycle shortly after their specification (Seki et al., 2007); and (3) PGCs undergo genome-wide epigenetic reprogramming (Seki et al., 2005; Seki et al., 2007; Hajkova et al., 2008).

Among the first genes to be expressed during PGC specification is *Blimp1* (B-lymphocyte-induced maturation protein 1, also known as *Prdm1*), a potent transcriptional repressor with a PR (PRDI-BF1 and RIZ) domain and five zinc fingers (Ohinata et al., 2005). *Blimp1* is first expressed in a small number of proximal epiblast cells, as well as in extra-embryonic cells, from E6.25. Lineage-tracing experiments have confirmed that Blimp1-expressing cells in the epiblast are lineage-restricted germ cell precursors (Ohinata et al., 2005). Mutations in *Blimp1* arrest PGC development early: only ~20 alkaline-phosphatase-positive cells (half the number found in wild-type animals) are detected in *Blimp1* mutant embryos, and these cells are quickly lost and do not populate the somatic gonad (Ohinata et al., 2005; Vincent et al., 2005). Remarkably, transcriptional profiling of *Blimp1*-deficient PGCs has shown that Blimp1 is responsible for the repression of virtually all downregulated transcripts during PGC specification (Kurimoto et al., 2008). This finding is consistent with the fact that Blimp1 is known to function as a potent transcriptional repressor in some somatic lineages (e.g. in the epidermal lineage) of the developing mouse. In these lineages, Blimp1 interacts with the Groucho family of co-repressors and with chromatin-modifying enzymes, including histone deacetylases and methyltransferases (Kallies and Nutt, 2007). Most of the targets of Blimp1 in somatic lineages, however, do not overlap with the genes that are repressed in PGCs, leaving open the issue of how Blimp1 selects its targets (Kurimoto et al., 2008). Clearly, unlike PIE-1 and Pgc, Blimp1 does not globally repress mRNA transcription, as many transcripts are upregulated during PGC specification in mice (Kurimoto et al., 2008).

Mouse PGCs remain positive for pSer2 and pSer5 during specification, but remarkably become negative for both phospho-epitopes later, during the migration of germ cells to the somatic gonad (between E8-E9) (Seki et al., 2007). At that time, PGCs also become arrested in the G2 phase of the cell cycle and begin to lose H3meK9 and to increase H3meK27 (both are transcriptionally repressive chromatin marks) (Seki et al., 2007). Bromo-UTP incorporation experiments have confirmed that mRNA synthesis is much reduced or absent during this time (Seki et al., 2007). Interestingly, ribosomal RNA transcription appears to remain active, as is observed in *C. elegans* and *Drosophila* (Seki et al., 2007; Seydoux and Dunn, 1997). Thus, in mouse PGCs, the global inhibition of RNAPII activity does not occur until a first wave of gene-specific transcriptional repression and activation has occurred. It is tempting to speculate that this difference might be due to the lack of pre-made germ plasm in mice (Fox et al., 2007). Indeed, among the early transcripts made by mouse PGCs are *nanos3* and *Dnd1*, the orthologs of which are inherited as germ plasm mRNAs in *Drosophila*, *C. elegans*, *Xenopus* and/or zebrafish (Mosquers et al., 1993; Subramaniam and Seydoux, 1999; Wang and Lehmann, 1991; Weidinger et al., 2003). Although the order of events differs somewhat between *Drosophila*, *C. elegans* and mice (Fig. 3) the parallels strongly suggest that transcriptional repression and chromatin remodeling are key to germ cell specification in many animals. In particular, the active repression of somatic programs appears to be an essential first step on the way to establishing germ cell fate.

A link between repression and activation?

Although we have learned much about the mechanisms that inhibit gene expression in the germline, we still understand little about the mechanisms that initiate the germline transcriptional program. Newly specified PGCs turn on the expression of many genes with evolutionarily conserved germline functions (i.e. the *nanos* family of translational repressors, and the *vasa* family of putative translational activators). In *C. elegans* and *Drosophila*, where *nanos* mRNA and other germline factors are provided maternally in the germ plasm, PGCs activate the transcription of these genes soon after inhibition of RNAPII is lifted and zygotic transcription commences (Kawasaki et al., 2004; Subramaniam and Seydoux, 1999; Van Doren et al., 1998). How is this program specified? Few transcription factors have been identified in germ cells, with the exception of those activated much later during gametogenesis (DeJong, 2006). In mammals, PGCs express the 'pluripotency'-related transcription factors Sox2, Nanog and Oct4, but these are also expressed in the epiblast and in ES cells, which do not express germline-specific transcripts. So what activates germline genes in PGCs? Paradoxically, recent evidence from mouse and *C. elegans* suggests that the same factors responsible for transcriptional repression may also be involved in activation.

Single-cell transcriptome profiling has shown that Blimp1 is required, not only for virtually all transcriptional repression, but also for the activation of ~50% of the genes that are upregulated in mouse PGCs. Genes that require Blimp1 for activation include those that show the highest specificity for PGC expression (e.g. *Nanos3*) (Kurimoto et al., 2008). The conclusion is that Blimp1 activity is required, directly or indirectly, to initiate part of the germline-specific transcriptional program, with another Blimp1-independent pathway also functioning in parallel (Kurimoto et al., 2008).

This second pathway appears to be controlled by Prdm14. Like Blimp1, Prdm14 is a PR domain protein that is expressed early in PGC development and is essential for PGC specification (Yamaji et al., 2008). Prdm14 expression in PGCs initially occurs independently of Blimp1, but becomes dependent on Blimp1 by E7.5. In the absence of Prdm14, presumptive PGCs (identified as cells that label positively for Blimp1) fail to downregulate GLP (G9a-related protein), the H3K9 methyltransferase, and, as expected, maintain H3K9me2 and do not upregulate H3K27me3. Prdm14-deficient PGCs also do not activate the pluripotency-related gene *Sox2*. Other aspects of PGC development, such as the downregulation of mesodermal genes and the activation of *Nanos3*, are not affected by the loss of Prdm14, indicating that it is required specifically to regulate genes that are involved in pluripotency and in epigenetic reprogramming (Yamaji et al., 2008). How Prdm14 and Blimp1 contribute to both the upregulation of certain genes and the downregulation of others is not known.

A dual positive/negative role has also been suggested for the MRG-1/MES system that inactivates the X chromosome in *C. elegans* germ cells. In somatic cells, loss of the histone deacetylase-containing co-repressor complex, NuRD, causes ectopic expression of germ plasm proteins and larval lethality (Unhavaithaya et al., 2002; Wang et al., 2005). Remarkably, these phenotypes can be suppressed by mutations in MRG-1 or in any one of the MES proteins, suggesting that the MRG-1/MES system contributes positively to the expression of germline genes, at least when they are mis-expressed in somatic cells that lack NuRD (Cui et al., 2006; Takasaki et al., 2007; Unhavaithaya et al., 2002; Wang et al., 2005) (Susan Strome, personal communication). Whether MRG-1 and the MES proteins normally function to activate the expression of germline genes, however, remains elusive. Experiments so far have revealed no obvious defects

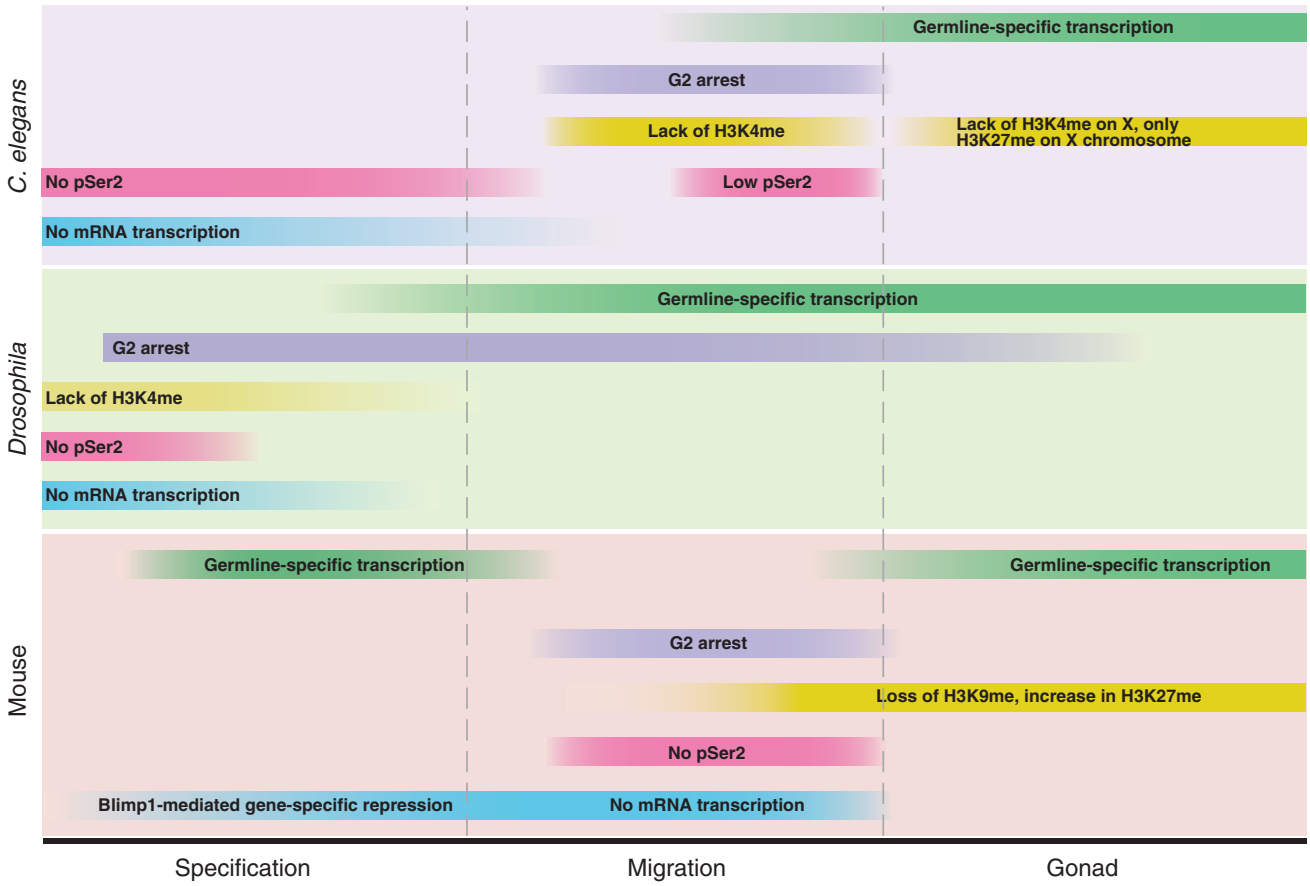

Fig. 3. Order of events during PGC specification. Comparison of the timing of different PGC events in *C. elegans*, *Drosophila* and mouse. For *C. elegans* (where PGCs do not migrate significantly), the 'migration' period refers to the period from the birth of Z2 and Z3 to hatching. During this time, Z2 and Z3 associate with the somatic gonad. The 'gonad' period refers to the period after hatching (L1 stage), when Z2 and Z3 start to proliferate.

in gene activation in *mrg-1* or *mes* mutant PGCs (Bender et al., 2006; Takasaki et al., 2007). One possibility is that the MES-2/MES-3/MES-6 complex functions redundantly with MES-4 and/or MRG-1 in activating genes. A microarray analysis of double mutant combinations might be needed to address this question.

A systematic analysis of promoters that are active in early germ cells has not been attempted as yet in any organism, but could be employed to identify the factors involved in transcriptional activation during PGC specification. A study in *C. elegans* has suggested that genes expressed in the germline are often found in or near sequence stretches that have a striking ~10-base periodicity of AA/TT dinucleotides (Fire et al., 2006). This periodicity biases the AA/TT nucleotides to one face of the helix and, in principle, could influence nucleosome positioning. It will be interesting to investigate whether promoters active in germ cells exist in a unique chromatin context that allows them to escape the repression experienced by the promoters of somatic genes.

Beyond transcription: the germ cell RNA world

After specification, PGCs begin the long journey to form the gametes. PGCs first migrate inside the embryo to join the somatic gonad, often associating with the intestine along the way (Fig. 1). Once in the gonad, they adopt a sexual identity, establish a stem cell pool (with the notable exception of female mammals), and eventually initiate meiosis and gametogenesis. Although we have highlighted in this review the extent of transcriptional repression and epigenetic reprogramming in PGCs, these mechanisms are unlikely to be the only ones, or even the primary ones, used by PGC descendents to regulate gene expression during differentiation. Several lines of evidence suggest that RNA-based mechanisms instead command center stage during germ cell differentiation. First, examples of post-transcriptional regulation are common in the germline and have been well documented, especially during gametogenesis (de Moor et al., 2005). A recent study in *C. elegans* that compared the regulatory contributions of promoters and 3′ UTRs found that 3′ UTRs are the primary source of spatiotemporal regulation for many genes expressed in the germline (Merritt et al., 2008). Second, many of the important regulators of germline development are RNA-binding proteins (Cinalli et al., 2008). Loss of these factors can cause dramatic effects on germline development. For example, in *C. elegans*, the simultaneous loss of two translational repressors causes germ cells to overproliferate and to adopt somatic cell fates, as if prematurely activating an embryonic-like program (Ciosk et al., 2006). Mutations in the RNA-binding protein Dead end similarly predispose mice to form differentiated tumors (teratomas) in their germline (Youngren et al., 2005). Finally, the RNA-rich germ granules are likely to represent a germline-specific platform for post-transcriptional regulation (Cinalli et al., 2008; Seydoux and Braun, 2006). In *Drosophila and C. elegans*, the germ granules are inherited with the germ plasm and remain in the

germline throughout most of germline development, with the exception of spermatogenesis (Seydoux and Braun, 2006). In mouse, related structures have been observed in PGCs by electron microscopy as early as E9 (Spiegelman and Bennett, 1973). For most of development, the germ granules are perinuclear and are often located close to nuclear pores, raising the possibility that many, if not all, germline mRNAs interact with germ granules upon exit from the nucleus (Eddy, 1975; Pitt et al., 2000). Most recently, germ granules have been implicated in the biology of small RNAs. Argonaute family members have been localized to germ granules in *Drosophila*, mice and *C. elegans* (Kotaja and Sassone-Corsi, 2007; Klattenhoff and Theurkauf, 2008; Batista et al., 2008; Wang and Reinke, 2008). piRNAs, a class of small RNAs specific to germ cells, are proposed to be produced in germ granules during gametogenesis (Klattenhoff and Theurkauf, 2008). In *C. elegans*, the germ granule components PGL-1 (P granule abnormality) and DEPS-1 (defective P granules and sterile) are required for small RNA-mediated interference (Robert et al., 2005; Spike et al., 2008).

Why such an emphasis on RNA regulation? One hypothesis is that the chromatin of germ cells is specialized to maintain most loci in a repressed but transcriptionally competent state. This state may be required to preserve the totipotency of the zygote, but may not be compatible with the DNA-based mechanisms commonly used by somatic cells to activate/repress genes during differentiation, forcing germ cells to use RNA-based mechanisms instead (Seydoux and Braun, 2006).

Conclusion

In this review, we have described how studies in *C. elegans*, *Drosophila* and mouse have all led to the view that transcriptional repression is essential to specify PGC fate. Although this paradigm provides an explanation for how germ cells escape somatic fates, it is not sufficient to account for the rich transcriptional program that is activated in PGCs. Many questions about this process remain: how do genes expressed in germ cells escape repression? What turns them on? How do chromatin and RNA regulation work together to promote gamete differentiation without compromising zygote totipotency? The realization that organisms as diverse as *C. elegans*, *Drosophila* and mouse use similar strategies to specify PGCs should encourage biologists working with other animals to join in the pursuit of revealing the many remaining secrets of the germline.

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