A germ-cell odyssey: fate, survival, migration, stem cells and differentiation

Meeting on Germ Cells

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The biennial meeting on Germ Cells at Cold Spring Harbor Laboratories took place during 9–13 October 2002. It was organized by Ruth Lehmann and David Page. The photograph is taken from the cover of the abstracts book and shows a *bag of marbles* mutant ovary that has germ cells labelled in green (anti-Vasa antibody) and somatic-cell membranes in orange (anti-spectrin antibody). Spectrin labelling also indicates the circular spectrosome in each undifferentiated germ cell. Photograph by Lilach Gilboa, from the laboratory of Ruth Lehmann.

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Introduction

The function of the germ line in all sexually reproducing organisms is to produce gametes that are able to contribute to the next generation (Fig. 1). Thus, like many a great epic film, the germ-cell saga spans generations and addresses such weighty themes as fate, immortality, death and transformation. During the recent meeting (9–13 October 2002) at Cold Spring Harbor Laboratories, germ cells did not disappoint. Well-known molecular ‘stars’ took their roles to new heights, and new ‘actors’ were introduced. Meeting participants gained an unprecedented overview of comparative germline development. Here, we focus on the comparison of some of the mechanistic and molecular characteristics of phylogenetic groups that were presented at this meeting, in the context of key steps in germline development.

Germline specification: ‘getting into character’

The germ-cell story begins and ends with ‘maternal pronucleus meets paternal pronucleus’. Soon after this encounter, the decision is made as to which cells assume a somatic fate and which potentially contribute to the next generation. So critical is this decision that, across diverse phyla, cells that are destined to take on germ-cell fates are physically separated from potential somatic cells early in embryogenesis, presumably to protect them from influences that would limit their potential or direct them along the path to a somatic fate.

One of the earliest recognized common themes in germline development among non-mammalian species is the presence of germline-associated cytoplasm (‘germ plasm’), which contains microscopically distinct, electron-dense granules. In some well-studied systems, such as *Drosophila melanogaster*, germ plasm in the embryo (known as ‘pole plasm’ in this species) can confer a germ-cell fate, whereas in other species, this ability does not exist or has not been shown. Even in species in which germ plasm or germ-associated granule localization has not been linked directly to the acquisition of a germ-cell fate, these granules segregate with the germ line and remain associated with it at later stages (Saffman & Lasko, 1999). Thus, studies that address germ-granule components and their assembly are of great interest, especially as some of these molecules are widely conserved across animal species.

At this meeting, several possible germ-granule components were introduced on the basis of their relationships with established components such as the VASA proteins. VASA proteins are germline DEAD-box RNA helicases that are found in primordial germ cells (PGCs) in species ranging from hydra to humans. P. Lasko (Montreal, Canada) provided a new piece of the *Drosophila* pole-plasm assembly puzzle in the form of Gustavus (Gus), which is a protein that is required for the localization of Vasa to the posterior of the developing oocyte. Lasko pointed out that there are homologues of Gus in evolutionarily distant organisms, including mammals (Styhler *et al.*, 2002). On a similar theme, K. Bennett (Columbia, MO, USA) presented members of three conserved protein families, CSN-5, KGB-1 and ZYX-1, that interact with the Vasa-related *Caenorhabditis elegans* GLH proteins (Smith *et al.*, 2002).

Nanos (Nos) and its related proteins have key roles in germ-cell fate acquisition, migration and survival in various species. Because *Drosophila* germ cells that lack *nos* die, it has been impossible to assess their fate. S. Kobayashi (Okazaki, Japan) circumvented this problem by using a deletion that removes three loci that are crucial for embryonic programmed cell death. Surprisingly, a small but significant percentage of *nos*⁻ germ cells incorporated into somatic tissues, and intermingled with and took on the appearance of their neighbouring somatic cells. In many systems, the physical mechanisms by which germinal particles are localized are unclear. M.L. King (Miami, FL, USA) proposed that the localization of the Nos-related *Xenopus laevis* protein Xcat2 into germinal particles occurs by a 'diffusion and entrapment' mechanism. A conserved zebrafish RNA-binding protein, Deadend, was introduced by J. Stebler (Göttingen, Germany). This protein colocalizes with Vasa and Nos in germ granules, and functional studies have suggested that it is crucial for the migration of PGCs and for the maintenance of their fate. Finally, three mouse homologues of Nos were revealed by M. Tsuda and colleagues (Mishima, Japan); two of these proteins are expressed and function in the germ line during embryogenesis.

In contrast to non-mammalian species, the determination of PGC fate in mammals is independent of germline-specific granules; it occurs instead through an inductive process. Because the precise nature of germ granules is unknown, the question remains as to whether they exist in mammals. Given that the factors that are associated with germ granules in non-mammalian species are also expressed in mammalian germ cells, it is tempting to speculate that all species share a basic germ-plasm machinery, and that this machinery exists in mammals as submicroscopic complexes that are rich in RNAs and RNA-binding proteins. One possibility is that this machinery could be assembled in response to inductive signalling by molecules such as bone morphogenetic protein 4 (BMP4; see below) and could function in a manner analogous to that of germ granules in model organisms, ensuring the formation and/or subsequent maintenance of germ-cell populations.

Non-conserved genes in early development

Unlike the conserved Vasa and Nos families, several important proteins involved in early germline development (in species such as worms, flies, frogs, fish and mammals) lack obvious orthologues in the genomes of species outside their phylogenetic group. In addition, some conserved proteins have different roles in different phyla. Given the striking parallels in germline development among various animals, one might expect conservation of protein families to be the rule. Thus, the reason for the existence of crucial group-specific

Fig. 1 | Summary of the main events in germline development in animals. The approximate temporal relationships between these events and the approximate stage of their occurrence within the context of overall development are shown. Details of germline development differ among species and between sexes within species and may not be consistent with this generalized diagram.

germline factors is unclear. Several possibilities were debated informally at the meeting, including that: rapid evolution of reproductive genes may be required for, or result from, speciation; group-specific factors might have evolved under group-specific developmental constraints; and constraints that shape the roles of various factors may be derived from the non-germline roles that these molecules have or once had during the course of evolution of a particular species. Alternatively, given some of the anatomical and mechanistic differences in germline development among the most studied groups, it may be equally surprising that any conservation exists across such large phylogenetic distances. Perhaps the conserved factors form a core machinery, the functions of which have been either maintained through evolution or modified for unique uses in each species. Regardless, functional studies of both group-specific genes and widely conserved genes are enriching the field of germ-cell research.

The set of important non-conserved germline components includes *Xenopus* *Xpat*, fly *oskar*, worm *pie-1* and mammalian *Oct4*. *Xpat* was originally identified as a transcript that localizes to the vegetal pole, and it encodes a protein that localizes to the germ plasm during the formation of stage 1 oocytes and during the period of PGC movement into the mesentery (Hudson & Woodland, 1998). Recent work discussed by H. Woodland (Coventry, UK) suggests that the *Xpat* protein has a role in targeting and/or transporting granules to germinal particles. In addition, *Xpat* is associated with centrosomes, although its precise function there is unknown.

G. Seydoux (Baltimore, MD, USA) discussed two general mechanisms for the asymmetrical distribution of germ granules: asymmetric enrichment before cell division, and degradation in nascent somatic blastomeres. PIE-1 normally segregates with germ-cell precursors and maintains germline fate. Video recordings of a *C. elegans* PIE-1::GFP (green fluorescent protein) fusion protein demonstrated its degradation in somatic blastomeres. Somatic PIE-1 degradation depends on the first of its two CCCH-type zinc fingers (ZF1) (Reese *et al.*, 2000) and on zinc-finger-interacting factor-1 (ZIF-1), a novel protein to which ZF1 binds. A yeast two-hybrid screen with ZIF-1 identified a potential E3 ubiquitin ligase subunit, Elongin C. Thus, ZIF-1 may provide a link between PIE-1

and a ubiquitin-mediated mechanism that ensures the degradation of germ-specific components in somatic cells during early development in *C. elegans*. Seydoux also made a comparison between *Drosophila* and *C. elegans*, pointing out the role of a conserved kinase, PAR-1, in relation to the non-conserved germ-plasm components Oskar and PIE-1, respectively (Pellerrieri & Seydoux, 2002). In both systems, PAR-1 is required to stabilize the germ-plasm proteins in the posterior of the embryo. In *Drosophila*, PAR-1 seems to stabilize Oskar by direct phosphorylation (Riechmann *et al.*, 2002). In *C. elegans*, PAR-1 seems to stabilize PIE-1 through two intermediates, MEX-5 and MEX-6, that must be downregulated in the posterior of the embryo for stabilization to occur (Cuenca *et al.*, 2003). This comparison illustrates the complex interplay between conserved and non-conserved factors in analogous processes.

The mouse protein Oct4 contains a DNA-binding POU domain and is expressed in the totipotent embryo, the germ line and undifferentiated embryonic stem (ES) cells. A. Tomilin (Freiburg, Germany) reported on a conditional knockout of the *Oct4* gene in PGCs. In *Oct4*⁻ PGCs, proliferation was halted between days E9.5 and E10, and the germ-cell population was virtually eliminated by apoptosis. The loss of *Oct4* in ES cells causes a different outcome—loss of pluripotency, not cell death as in PGCs—so Tomilin suggested that Oct4 has different targets during development.

As noted by M.A. Surani (Cambridge, UK), because there is no evidence for the presence of germline determinants in mammals, and only a subset of BMP4-responding cells go on to acquire a germ-cell fate, other factors must be required for germline determination. Surani and colleagues therefore compared gene expression between single PGC founders and their somatic neighbours that share a common ancestry, and identified *fragilis*, a gene that encodes an interferon-inducible transmembrane protein. Its expression is dependent on the dose of BMP4 and the protein may have a role in establishing germ-cell competence. They also identified *stella*, a gene that is first expressed in the nascent PGCs, starting at the centre of the *fragilis*-positive region, and thereafter in migrating PGCs. A key finding is that, unlike somatic cells, nascent PGCs repress region-specific *Hox* genes such as *Hoxb1*. Thus, the expression of *stella* and *Hoxb1* is mutually exclusive in germline and somatic neighbours (Saitou *et al.*, 2002). Y. Matsui (Osaka, Japan) and colleagues used a primary culture system of mouse epiblast cells, from which PGCs arise, to show that inductive signals from the extra-embryonic ectoderm are required between days E5.25 and E5.5; inductive signalling with BMP4 alone stimulated PGC formation at day E5.5 but not before that stage. The investigators also identified *mil-1* and *mil-2* by virtue of their differential expression between early and migrating PGCs. Both genes encode members of a family of interferon-induced transmembrane proteins, one of which is identical to *fragilis* (Tanaka & Matsui, 2003).

The analysis of early events in mammalian germline development is enhanced by new germ-cell markers such as the *Oct4::GFP* fusion presented by C. Wylie (Cincinnati, OH, USA; Anderson *et al.*, 2000; Molyneaux *et al.*, 2001). The use of this marker was shown in the analysis of mouse germ cells that lack the pro-apoptotic gene *Bax*. Similar to the ectopic localization of PGCs that lack *nos* and other cell-death genes in *Drosophila*, mammalian germ cells that survived early fetal development were found in ectopic, somatic locations, but still expressed germ-cell markers.

To differentiate or not to differentiate

Once germ-cell fate is specified, germ cells must find, interact with and populate the developing somatic gonad (Starz-Gaiano & Lehmann, 2001). In organisms that produce germline stem cells, either all or a subset of the germ cells that enter the somatic gonad become stem cells. In the latter case, what determines which cells acquire (or maintain) stem-cell potential? The results from several laboratories suggest that this decision is regulated both spatially and temporally. M. Asaoka, from the laboratory of H. Lin (Durham, NC, USA), presented a lineage analysis in *Drosophila* using GFP-marked pole cells to distinguish between two possible mechanisms of stem-cell specification from the PGC pool: lineage dependent versus position dependent. Position in the embryonic gonad appeared to be critical. D. Godt (Toronto, Canada) discussed the role of Traffic jam, a transcription factor from the musculo-aponeurotic fibrosarcoma (Maf) family. In *traffic jam* mutants of *Drosophila*, the cells of the somatic gonad and the germ line do not intermingle correctly, and the premature separation of germ cells and somatic cells blocks the proper differentiation of both cell types. Data presented by E.J. Hubbard (New York, NY, USA) suggested that the timing and position of the developmentally earliest meiotic entry in the *C. elegans* hermaphrodite is determined by an interaction between the germ line and the early-larval proximal somatic gonad, in addition to the well-characterized interaction with the distal somatic gonad. An investigation of the PTEN lipid phosphatase in the mouse male germ line by T. Nakano (Osaka, Japan) suggests that this protein is crucial for the regulation of PGC proliferation. Male germ cells that lack PTEN fail to arrest properly at the G1 stage on days E13.5–E14.5, resulting in an increased number of bilateral testicular teratomas. It was proposed that, in the absence of PTEN, PGCs may de-differentiate into pluripotent ES or embryonic-germ-like cells, suggesting that PTEN is a crucial regulator of germ-cell differentiation (Kimura *et al.*, 2003).

Once germline stem cells are established, their self-renewal and differentiation must be regulated. If differentiation exceeds self-renewal, the germ line is depleted, and if self-renewal exceeds differentiation, overproliferation may occur. Regulation of germline proliferation involves input from neighbouring somatic cells, although different signalling pathways are used for analogous purposes in different organisms. Often, there is an anatomical axis of proliferation and differentiation, which has stem cells at one end and mature gametes at the other (open) end (Fig. 2). For example, in both sexes of *Drosophila*, in *C. elegans* and in male mammals, localized somatic cells signal to the germ line, and germline-localized factors respond. The molecular details of these signalling pathways are now under investigation in several systems.

In *Drosophila* males, the Jak/Stat pathway has been implicated in the maintenance of germline stem-cell fate through a 'hub-to-germline' signal (Kiger *et al.*, 2001; Tulina & Matunis, 2001). Work presented by E. Matunis (Baltimore, MD, USA) suggests that the Jak/Stat signalling pathway may also act in the hub itself, where it is activated in an autocrine manner by the ligand Unpaired, to regulate the size of the hub and the number of germ cells that it contacts. M. Fuller (Stanford, CA, USA) presented a cell-biological approach, suggesting that the orientation of stem-cell divisions is crucial for determining the fate of the two daughter cells. Mutations in the gene that encodes centrosomin, an integral centrosome component, result in spindle-orientation defects that correlate with an increased number of germline stem cells. In *Drosophila* females, a BMP homologue, the product of the *decapentaplegic* gene, functions as the main

Fig. 2 | A schematic representation of gonad anatomy in (left to right) the *Drosophila* male, *Drosophila* female, *Caenorhabditis elegans* hermaphrodite and male mammal. This diagram emphasizes the relative positions of stem cells (green) and differentiated germ cells. The green-to-yellow arrow indicates the direction of proliferation to differentiation. In each case, the direction of the arrow corresponds to an anatomical axis that can be described as ‘apical-tip to basal-lumen’ and the ‘anterior-to-posterior’ axes in the *Drosophila* male and female, respectively, ‘distal-to-proximal’ in *C. elegans*, and a radial axis of ‘basal-lamina-to-lumen’ in male mammals. Somatic cells that are important for stem-cell maintenance are shown in red.

soma-to-germ-line signal that maintains stem cells (Spradling *et al.*, 2001). The *bag of marbles* (*bam*) gene is required for the differentiated germ-cell fate and antagonizes stem-cell fate. D. McKearin (Dallas, TX, USA) presented evidence that a transcriptional silencer element is active specifically in stem cells and prevents *bam* expression, blocking differentiation and allowing the maintenance of stem cells (Chen & McKearin, 2003). On the basis of an analysis of mutants in the *zero population growth* gene, which encodes a gap-junction protein (Tazuke *et al.*, 2002), and of observations from wild-type flies, L. Gilboa (New York, NY, USA) suggested the existence of an intermediate (pre-cystoblast) population of cells. She further suggested that pre-cystoblasts are probably an intermediate in the differentiation process rather than a ‘transit-amplifying’ cell population, and that germ-cell tumours observed in *bam* mutants (or under similar conditions) may primarily contain pre-cystoblasts rather than stem cells.

In *C. elegans*, GLP-1, a Notch-family receptor, is activated in the germ line in response to a somatically produced ligand to promote mitosis and/or inhibit meiosis. The RNA-binding protein GLD-1 acts genetically downstream of, and in opposition to, the activity of the GLP-1-mediated pathway. GLD-1 and GLD-2, an atypical poly(A) polymerase, are redundant in this meiosis-promoting role. In some way, these—and probably other—components demarcate the mitosis–meiosis boundary at the proximal edge of the stem-cell population. Although the picture is incomplete, a complex web of regulation that involves several RNA-based controls is emerging. The results from a genetic strategy presented by T. Schedl (St Louis, MO, USA) suggest that the *nos*-related gene *nos-3* is redundant with *gld-2* in promoting meiosis. Analysis of GLD-1 protein levels revealed that part of the spatial mechanism that determines the position of the mitosis–meiosis border involves inhibition of GLD-1 levels by *glp-1* and elevation of GLD-1 by *gld-2/nos-3*. J. Kimble (Madison, WI, USA) presented an analysis of GLD-2 and its activating protein GLD-3,

which is another RNA-binding molecule. These studies led to the proposal that meiotic RNAs are inactive and that poly(A) addition by GLD-2/GLD-3 enables translation. The plot is thickened further by the redundant activity of the PUF (PUMILIO and FBF) family members FBF-1 and FBF-2, which maintain stem-cell proliferation in late-larval and adult stages and which interact physically with GLD-3 and NOS-3 (Crittenden *et al.*, 2002; Eckmann *et al.*, 2002; Kraemer *et al.*, 1999).

Other reports reflected the importance of regulating germline development through the control of RNA in many phyla. R. Reijo Pera (San Francisco, CA, USA) brought the PUF family into the mammalian germline picture with her report on the deleted in azoospermia (DAZ) gene family and interacting factors (Moore *et al.*, 2003). Previous work had shown that DAZ genes are required for germ-cell development in diverse organisms, ranging from worms to humans. In recent studies, PUM2, a human PUF family protein, was identified as one of six DAZ-interacting proteins. PUM2, and several other interacting proteins, are expressed specifically in ES cells, PGCs and mature germ cells in both sexes. All six proteins contain RNA-binding motifs. J. Richter (Worcester, MA, USA) described findings from mice that were homozygous for a null mutation of *CPEB* (cytoplasmic-polyadenylation-element-binding protein). Although ovaries initially form and contain numerous immature germ cells, as these cells enter meiosis, the oocyte chromatin becomes diffuse and messenger RNAs that encode synaptonemal-complex proteins are not correctly polyadenylated or translated (Tay & Richter, 2001). Studies presented by M. Hengartner (Zurich, Switzerland) revealed a role for a CPEB-related protein in cell death in the *C. elegans* hermaphrodite germ line. Thus, CPEB family members are continuing to be found throughout the animal kingdom in various germline roles (Mendez & Richter, 2001).

Transcriptional control, silencing and epigenetics

Germ cells are the vehicles through which DNA is passed from one generation to the next. What molecular mechanisms underlie the ability of germ cells to resist somatic fates, and yet undergo a dramatic differentiation to gametes, while maintaining their ability to contribute to the formation of the totipotent zygote? Transcriptional and epigenetic controls that govern maintenance and reprogramming of the germ line in diverse organisms are of increasing interest as they may hold the key to some of these questions (Sassone-Corsi, 2002; Surani, 2001; Pirrotta, 2002). In *Drosophila* and *C. elegans*, transcriptional silence is a hallmark of nascent germ cells. Evidence that transcriptional silence may be a characteristic of vertebrates was presented by M.L. King. The expression of zygotic genes in nascent *Xenopus* PGCs was assessed by RNA PolII CTD phosphorylation, and was shown to start at the neurula stage. These data suggest that the failure of PGCs to adopt endodermal fates, despite the fact that they inherit endodermal determinants such as VegT, may be due to transcriptional repression in nascent PGCs at a time when VegT targets would normally be activated. Transcriptional silence in the early *Drosophila* germ line was discussed by T. Jongens (Philadelphia, PA, USA) in the context of the gene *germcell-less* (*gcl*). The removal of *gcl* activity results in both a failure to establish transcriptional silencing in the germ line–destined nuclei and a reduction in the number of pole cells formed, whereas ectopic GCL expression causes an ectopic decrease in transcriptional activity. Thus, transcriptional quiescence seems to be imposed before pole-cell formation (Leatherman *et al.*, 2002). GCL-interacting factors include PHO-like, a protein with a zinc-finger domain that is similar to PHO, a member of the Polycomb group (see next paragraph). Jongens presented a model in which GCL may tether chromatin to the nuclear envelope, thereby silencing transcription in a manner similar to telomeric silencing. R. Martinho (New York, NY, USA) reported that *tailless* (*tll*) and *zerknüllt* (*zen*) in *Drosophila*, two genes that are normally transcriptionally repressed before and during gastrulation, are not repressed in the absence of the non-coding *polar granule component* RNA in early embryonic germ cells. Furthermore, Osa, a component of the Swi/Snf chromatin-remodelling protein complex, is required for repression of *zen* transcription in germ cells during gastrulation, suggesting that several independent but sequential mechanisms act to repress transcription in early germ cells.

In *C. elegans*, at least two systems of germline silencing are at work: the PIE-1 system, which represses transcription in early germline blastomeres until the ~100-cell stage, and the MES system, which is named for the maternal effect sterile (*mes*) mutants that led to its discovery (Seydoux & Schedl, 2001; Pirrotta, 2002). Several *mes* genes share homology with genes of the fly Polycomb group, which are known for their role in the repression of homeotic genes. Several laboratories (Fong *et al.*, 2002; Kelly *et al.*, 2002) have shown germline repression of chromatin on the X chromosome and its correlation with certain histone modifications. MES proteins participate in this silencing. L. Bender (Bloomington, IN, USA) reported on SET-2, one of several SET-domain proteins that are involved in MES-mediated silencing, suggesting that this protein marks active chromatin. C. Bean (Atlanta, GA, USA) elaborated on recent findings, suggesting that the *C. elegans* paternal X chromosome is preferentially inactivated in early XX embryos. A model was suggested in which an epigenetic imprint is established in the male germ line, and its decay in the early embryo regulates the onset of somatic dosage

compensation. Remarkably, mammalian dosage compensation can involve non-random X-chromosome inactivation limited to the paternally inherited X chromosome (in marsupials and the extra-embryonic tissues of eutherian mammals). Because both processes may involve proteins that are related to Esc (extra sexcombs) in *Drosophila*, MES-6 in worms and Eed (embryonic ectoderm development) in mouse, it is tempting to speculate that an ancient large-scale silencing system may have been co-opted by diverse phylogenetic groups to establish and/or maintain active and inactive chromatin states over large contiguous stretches of the genome (Pirrotta, 2002).

Although global silencing in germ cells has not been demonstrated in mammals, epigenetic reprogramming is known to occur in each generation. A better understanding of these processes is becoming especially important as issues are raised about problems in animal cloning and in therapeutic cloning for humans. In a compelling talk by R. Jaenisch (Cambridge, MA, USA), these processes were discussed with respect to cloning by somatic-cell nuclear transfer. Jaenisch and colleagues addressed the questions: Does the tissue source of the nucleus matter in nuclear transfer? And are common problems in cloned embryos, such as large size and defects in organogenesis, of genetic or epigenetic origin? Using nuclear transfer in mice, they found that ES-cell-derived clones had better survival rates than clones derived from mature somatic cells. Furthermore, the group showed that problems with nuclear transfer are likely to be epigenetic rather than genetic in origin. Strikingly, 4–5% of all genes, but 30–50% of imprinted genes, were aberrantly expressed in cloned embryos. Jaenisch concluded that the lengthy processes of normal gametogenesis and fertilization may give the zygotic nucleus the ability to direct normal development, whereas somatic-cell nuclear transfer may not provide the same opportunity (Hochedlinger & Jaenisch, 2002; Humpherys *et al.*, 2002).

And the ‘Oskar’ goes to...

Although they cannot be covered here, several other themes emerged at this meeting, including molecular and evolutionary aspects of meiosis, gametogenesis, germline sex determination, sex-chromosome evolution, sperm–female (both sperm–egg and sperm–soma) interactions, differential gamete success, the evolution of reproductive proteins, the trade-off between male reproductive success and female health in *Drosophila*, and the astonishing (if not alarming) effects of the bacterium *Wolbachia* on reproduction and sex determination. In short, there is every indication that more surprises, insights and potentially beneficial discoveries are in store as we explore the nature of germ cells in different organisms.

Despite a host of worthy nominees, the unofficial consensus was that the ‘Oskar for best picture’ of the 2002 Germ Cell Meeting goes to “GFP-tagged zebrafish germ cells” (E. Raz, Göttingen, Germany).

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