polyhomeotic is required for somatic cell proliferation and differentiation during ovarian follicle formation in *Drosophila*

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Summary

The *polyhomeotic* (*ph*) gene of *Drosophila* is a member of the Polycomb group (*Pc*-G) genes, which are required for maintenance of a repressed state of homeotic gene transcription, which stabilizes cell identity throughout development. The *ph* gene was recovered in the course of a gain-of-function screen aimed at identifying genes with a role during ovarian follicle formation in *Drosophila*, a process that involves coordinated proliferation and differentiation of two cell lineages, somatic and germline. Subsequent analysis revealed that *ph* loss-of-function mutations lead to production of follicles with greater or fewer than the normal number of germ cells associated with reduced proliferation of somatic prefollicular cells, abnormal prefollicular cell encapsulation of germline cysts and an excess of both interfollicular stalk cells and polar cells. Clonal analysis showed that *ph* function for follicle formation resides specifically in somatic cells and not in the germline. This is thus the first time that a role has been shown for a *Pc*-G gene during *Drosophila* folliculogenesis. In addition, we tested mutations in a number of other *Pc*-G genes, and two of them, *Sex combs extra* (*Sce*) and *Sex comb on midleg* (*Scm*), also displayed ovarian defects similar to those observed for *ph*. Our results provide a new model system, the *Drosophila* ovary, in which the function of *Pc*-G genes, distinct from that of control of homeotic gene expression, can be explored.

Key words: Oogenesis, *polyhomeotic*, Polycomb group, follicle cells, *Drosophila*

Introduction

Drosophila melanogaster oogenesis provides an outstanding opportunity for studying several fundamental aspects of developmental biology (for reviews, see King, 1970; Spradling, 1993; Spradling et al., 1997). Indeed, ovarian follicle formation in *Drosophila*, involving coordinated regulation of proliferation, differentiation and morphogenesis of two distinct cell lineages, somatic and germline, occurs throughout adult life through continuous activity of somatic and germline stem cells, and is thus readily accessible for observation and analysis. Each ovary is composed of 12 to 16 ovarioles, which represent linear strings of maturing follicles, also called egg chambers. Each follicle contains a single oocyte developing independently of its neighbors within a somatic follicular epithelium. Germline and somatic stem cells reside in the germarium, at the anterior end of each ovariole (Fig. 1). At the most anterior part of the germarium, overlying somatic cells (terminal filament and cap cells) create a niche that maintains the germline stem cell population via secretion of several signaling molecules (Spradling et al., 2001; Lin, 2002). Oogenesis starts with the asymmetric division of a germline stem cell, which generates both a daughter stem cell and a differentiated daughter cell, the cystoblast. Within region 1 of the germarium (Fig. 1), each cystoblast undergoes exactly four synchronous divisions with incomplete cytokinesis to give rise to a germline cyst comprised of 16 cystocytes connected to each other by cytoplasmic bridges called ring canals. One of the two cells with four ring canals becomes an oocyte and proceeds through meiosis, while the remaining 15 cystocytes develop as nurse cells and begin endoreplication of their DNA. The population of germline cysts during these stages is surrounded by somatic inner sheath cells (Margolis and Spradling, 1995). Morphologically unidentifiable somatic stem cells reside in the region 2a/2b boundary of the germarium and give rise to mesenchymal somatic cells (or prefollicular cells) invaginating from the germarium wall to individuate each germline cyst (Fig. 1). This migration is associated with a characteristic transformation of the germline cyst, consisting of both flattening and lengthening of the cyst so that it spans the width of the germarium. Part of this population of prefollicular cells continues to divide and differentiates into a monolayer of polarized epithelial follicle cells (Margolis and Spradling, 1995). The most mature cyst in the germarium and its surrounding follicular cells form a stage 1 follicle in region 3 (Fig. 1). Anteriorly to this chamber, other prefollicular cells interdigitate to form an interfollicular stalk of five to seven cells that separates the newly born follicle from the rest of the germarium and persists between all the maturing follicles of the ovariole. Prefollicular cells give rise to a third population of specialized somatic cells, the polar cells, which represent pairs of cells defining the anterior and posterior poles of each follicle (Fig. 1). Lineage studies have previously demonstrated

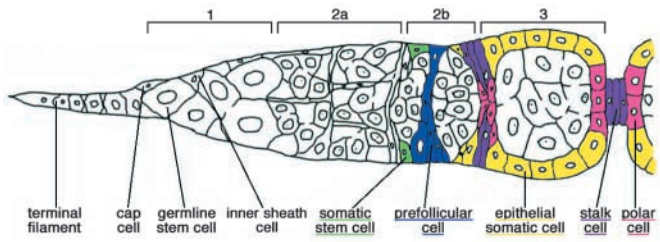

Fig. 1. The *Drosophila* germarium. Schematic drawing of the *Drosophila* germarium, modified from Zhang and Kalderon (Zhang and Kalderon, 2001). Germarial regions 1, 2a, 2b and 3 are indicated above the drawing. At the most anterior part of the germarium, overlying somatic cells (terminal filament and cap cells) create a niche to maintain the germline stem cells. Anterior region 1 contains two to three germline stem cells (GSCs); GSC division is asymmetric and generates both a daughter stem cell and a differentiated daughter cell called a cystoblast. Each cystoblast undergoes four rounds of mitosis with incomplete cytokinesis to produce a syncytium of 16 cystocytes, the germline cyst. One of these 16 cells becomes the oocyte, while the remaining 15 cells develop as nurse cells. The population of germline cysts during these stages is surrounded by somatic inner sheath cells. In region 2a/2b, approximately two somatic stem cells (SSCs), represented in green, give rise to a population of undifferentiated cells, the prefollicular cells, in blue, which migrate to envelop each germline cyst individually. These cells diverge soon after to give rise to: two pairs of polar cells, in pink, which mark the anterior and posterior poles of the follicle; interfollicular stalk cells, in purple, which are responsible for follicle budding from the germarium; and epithelial follicular cells, in yellow, which form a polarized epithelium around each follicle.

that precursors for stalk and polar cells separate from the general follicle cell lineage within germarial region 2b, although specific markers distinguishing these cell types can be visualized only once follicle budding is complete (Tworoger et al., 1999).

Ovarian follicle formation thus requires the coordinated control of proliferation and differentiation of both germline and somatic cells. Regulation of somatic cell proliferation and patterning has been shown to involve both soma–soma and germline–soma intercellular signaling. For instance, the apically located somatic terminal filament and cap cells are a source of Hedgehog (Hh) and Wingless (Wg) signals, both regulating somatic stem cell proliferation and/or maintenance (Forbes et al., 1996; Zhang and Kalderon, 2000; Zhang and Kalderon, 2001; Song and Xie, 2003). Hh has been shown to play an additional role in prefollicular cell differentiation and morphogenesis (Besse et al., 2002). Mature 16-cell germline cysts also instruct prefollicular cell morphogenesis during cyst encapsulation, since, in the absence of germline cells, prefollicular cells do not undergo any cell shape changes (Spradling et al., 1997). Indeed, both secreted proteins (Brainiac, Egghead, Gurken/TGF-α) encoded by genes with germline-specific function and transmembrane proteins expressed in prefollicular cells (Torpedo/EGFR) are thought to act as components of germline-to-soma signaling pathways required for correct encapsulation of mature 16-cell cysts by prefollicular cells (Goode et al., 1992; Goode et al., 1996a; Goode et al., 1996b; Rubsam et al., 1998). In addition, specification of polar cells and interfollicular stalk cells involves germline-to-soma Delta/Notch signaling. If *Notch* function is removed in somatic cells, or that of *Delta* in germline cells, no polar cells are specified (Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001). In the absence of polar cells, the separation of germline cysts by somatic cells is defective, and this leads to accumulation of several cysts in the same follicle (Grammont and Irvine, 2001). Polar cells also seem to be a source of signaling molecules necessary for specification of interfollicular stalk cell fate. Delta/Notch signaling may be involved, since induction of mutant *Delta* clones in the somatic cells leads to an absence of interfollicular stalks (Lopez-Schier and St Johnston, 2001). In addition, JAK/STAT signaling is required. Lack of Stat92E or Hopscotch (the *Drosophila* JAK homolog) in somatic cells leads to an absence of interfollicular stalk cells and to an increase in the number of polar cells, whereas overexpression of Unpaired leads to an increase in the number of stalk cells and to an absence of polar cells (Baksa et al., 2002; McGregor et al., 2002).

In the course of a gain-of-function screen aimed at identifying genes with somatic function during oogenesis, we found that overexpression of *polyhomeotic* (*ph*) leads to a significant reduction in female fecundity. The *ph* gene is a member of the Polycomb group (*Pc*-G) genes. Genes of the *Pc*-G and the *trithorax*-group (*trx*-G) have been shown to control early embryonic development, as well as imaginal disc morphogenesis, through maintenance, but not initiation, of the spatial pattern of homeotic and segment polarity gene expression during embryonic and imaginal disc development (Dura and Ingham, 1988; Ingham and Martinez-Arias, 1992; Simon et al., 1992; Paro, 1993; Randsholt et al., 2000; Francis and Kingston, 2001). Several lines of evidence indicate that *Pc*-G proteins associate in multimeric complexes associated with chromatin, thereby ensuring gene silencing throughout development (Fauvarque and Dura, 1993; Chan et al., 1994), while *trx*-G proteins counteract the silencing effect of Pc-G proteins (Poux et al., 2002). Mutations in *Pc*-G genes exhibit synergistic and dose-dependent effects, suggesting cooperative interactions between proteins encoded by these genes. In addition, each *Pc*-G protein recognizes approximately 100 sites on polytene chromosomes from *Drosophila* salivary glands, but these sites do not fully overlap between these proteins (Zink and Paro, 1989; DeCamillis et al., 1992; Lonie et al., 1994). Two-hybrid and immunoprecipitation studies have shown that the *Pc*-G proteins form at least two non-overlapping protein complexes. One complex, named PRC1, includes Polycomb (Pc), Posterior Sex Combs (Psc), Polyhomeotic (PH), dRing/Sex Combs Extra (Sce) and Sex Comb on Midleg (Scm) (Shao et al., 1999; Saurin et al., 2001; Fritsch et al., 2003), while the other contains Extra Sex combs (Esc) and Enhancer of zeste (E(z)) (Ng et al., 2000; Tie et al., 2001). Nevertheless, it has been shown that Pc, Psc and PH are differentially distributed on regulatory sequences of the *engrailed*-related gene *invected*, suggesting that there may be multiple Pc-G protein complexes with different compositions that function at different target sites (Strutt and Paro, 1997). Since some Pc-G proteins are not part of these complexes, for example Polycomb-like (Pcl), it is supposed that there are other as yet unidentified complexes of Pc-G proteins (for a review, see Otte and Kwaks, 2003).

The *ph* locus is located on the X chromosome at 2D2-3 position and consists of two different transcription units,

termed *polyhomeotic-distal* (*ph-d*) and *polyhomeotic-proximal* (*ph-p*), which correspond to a tandem duplication of DNA (Dura et al., 1987; Deatrick et al., 1991). Mutants homo- or hemizygous for lesions in either one of the two units display the same phenotypes during embryonic development, suggesting that these two molecular units encode redundant functions (Dura et al., 1987). Furthermore, inactivation of one unit is transcriptionally compensated by the other, thereby maintaining a nearly wild-type level of *ph* product (Fauvarque et al., 1995).

We show here that overexpression of the *polyhomeotic* gene leads to specific defects in follicle formation. Complementary analysis revealed that *ph* function is required within germarial somatic cells for their differentiation and proliferation. Removal of *ph* function leads to production of follicles with more or less than 16 germ cells, abnormal accumulation of cysts in the germarium, expansion of the interfollicular stalks between adjacent follicles, and polar cell differentiation defects. In addition, loss-of-function mutations reduce prefollicular and follicular cell proliferation in the germarium. Finally, we show that two other *Pc*-G members, *Sce* and *Scm*, are also implicated in early oogenesis.

Materials and methods

Somatic gain-of-function screen

The mutagenesis was performed using standard methodology, by mobilizing a P{y+}UAS transposon [http://flybase.bio.indiana.edu/.bin/fbidq.html?FBrf0093303 (J. Merriam)] located on the X chromosome in a *yw* genetic background. This insert has UAS sequences and the *hsp70* basal promoter oriented to transcribe leftward out of the 5′ P end. In total, 2500 independent insertions were generated (French Consortium, a part of the lines was published in Monnier et al., 2002). Males from each resulting line were crossed to *w; da-GAL4* females (Wodarz et al., 1995), which provide the GAL4 protein under the control of the ubiquitous *daughterless* promoter. Flies were raised at 25°C on standard media. The fecundity of the female progeny was first analyzed by counting the number of eggs laid per female and per day. Ovaries from selected lines were then dissected, stained with a nuclear marker (DAPI), and tested for their morphology. Since the vector used to generate P{y+}UAS lines was a pUAST construct, this GAL4/UAS system allows overexpression of genes only in the somatic line (Rorth, 1996).

We isolated by reverse PCR the genomic sequences flanking the selected P{y+}UAS insertions. Genomic DNA was extracted, digested by MspI and then circularized by ligation. The PCRs were performed under the following regime: (1) denaturation at 92°C for 5 minutes; (2) 40 cycles at 89°C for 5 seconds, 92°C for 10 seconds, 51°C for 45 seconds and 72°C for 2 minutes; and (3) a final 2-min extension at 72°C. The primer sequences correspond to the 5′ P end. Primer sequences used to amplify the fragment were: OUY311 (5′-TTGATTCACTTTAACTTGCAC-3′) and OUY521 (5′-ACACAA-CCTTTCCTCTCAACAA-3′). The PCR products were used directly in a nested PCR with the following primer sequences: OUY521 and OUY32 (5′-GCTTTCGCTTAGCGACGTG-3′). The cycle conditions were: (1) 92°C for 2 minutes; (2) 20 cycles at 89°C for 5 seconds, 92°C for 10 seconds, 56°C for 45 seconds and 72°C for 2 minutes; and (3) 72°C for 2 minutes. Amplification products were then sequenced directly by using the primer sequence OUY53 (5′-ATACTTCGGTAAGCTTCGGCTATCGACG-3′).

Drosophila stocks

The control fly strains used were: w^c and w^{1118}. The ph^{504} (noted ph^0) amorphic allele carries lesions in both *ph-p* and *ph-d* units (Dura et al., 1987). All other *ph* alleles are hypomorphic alleles. The ph^{lac} strain was induced by *PlacW*-element mutagenesis and corresponds to an insertion into the proximal unit, close to the first intron-splicing site (Fauvarque et al., 1995). The A101 enhancer-trap insertion (Ruohola et al., 1991) was used for tissue-specific β-galactosidase staining of polar cells. For testing *ph* overexpression, *patched-GAL4* (Bloomington Stock number 2017), *da-GAL4* (Wodarz et al., 1995), *e22c-GAL4* (Bloomington Stock number 1973) and *hs-GAL4* (Bloomington Stock number 3738) lines were used in combination with *PH-C7* (gift from F. Maschat), *m20* and *m35* (Netter et al., 2001) P[UAS:cDNA *ph*] lines. Flies were raised on standard media at 25°C.

Clonal analysis

Induction of Flipase expression by heat shock was done using either an X-chromosomal or a II chromosomal *hs-flp* construct by heat shocking females just after the eclosion at 38°C for 1 hour, once. Somatic overexpression of *ph* was performed by generating Flip-out/GAL4 clones in females *hs-flp/+; UAS-*ph^x*/Act>CD2>Gal4 UAS-GFP* (Pignoni and Zipursky, 1997; *UAS-*ph^x denotes either *PH-C7*, *m20* or *m35*). Flies were dissected 8 or 9 days after eclosion. Clones were detected by the presence of *GFP* expression. Mutant clones for *ph* were generated by mitotic recombination using the FLP/FRT system (Xu and Rubin, 1993) in females *hs-flp tub-lacZ FRT101/* ph^{504} *w FRT101* or *ubi-nls-GFP FRT101/* ph^{504} *w FRT101*; *hs-*flp^{38}*/+*. *hs-flp tub-lacZ FRT101* and *ubi-nls-GFP FRT101; hs-flp38* lines were gifts from S. Goode (unpublished) and A. Guichet, respectively; the ph^{504} *w FRT101* was previously described (Beuchle et al., 2001). Flies were dissected 2, 4 or 8 days after eclosion. Clones were detected by the loss of *lacZ* or *GFP* expression. FRT-mediated recombination events were induced specifically in the germline using an *ovo-flp* transgene and revealed by loss of nuclear *GFP* expression (I. Brun and C. Desplan, unpublished results) in *ubi-nls-GFP FRT101/* ph^{504} *w FRT101; ovo-flp/+* females. Flies were dissected 4 or 8 days after eclosion. Mutant clones for other *Pc*-G genes were also generated and the following strains were used: *w; FRT82B* Scm^{D1}*/TM6B, w; FRT82B* Sce^1*/TM6B, yw; FRT42D Su(z)*$2^{I.b8}$*/SM6b, yw FRT42D* Pcl^{D5}*/CyO, yw; FRT42B sca* Asx^{XT129}*/CyO, yw hs-flp; FRT42B hs-nGFP* (Beuchle et al., 2001), *yw hs-flp; FRT42D ubi-nls-GFP/CyO* and *yw hs-flp; FRT82B ubi-nls-GFP* (gift from A. Guichet). Asx^{XT129} and Pcl^{D5} have been reported to be null mutations, *Su(z)*$2^{I.b8}$ is a deficiency that removes *Psc* and *Su(z)2* (Soto et al., 1995), Sce^1 has been reported to be a null mutation (Breen and Duncan, 1986) and Scm^{D1} is a frameshift mutation that genetically behaves as a null allele (Bornemann et al., 1998). Flies were dissected 8 or 9 days afer eclosion. In all cases, flies were kept at 25°C on standard media.

Staining ovaries

For antibody staining, ovaries were dissected in PBS and fixed in 3:1 heptane:4% formaldehyde in PBT (0.1% Tween-20 in PBS) or in 4% paraformaldehyde in PBT for 25 minutes. The ovaries were washed three times in PBT, blocked in 2% BSA for 1 hour, then incubated with primary antibody for 3 hours at room temperature or at 4°C overnight. The primary antibodies used in this study were rabbit polyclonal anti-β-galactosidase (1:200, Boehringer), mouse monoclonal anti-β-galactosidase 40-1a [1:200, Developmental Studies Hybridoma Bank (DSHB)], mouse monoclonal anti-Orb 6H4 (1:30, DSHB), mouse monoclonal anti-Fasciclin III 7G10 (1:10, DSHB), rabbit polyclonal anti-α-Spectrin (1:1000) (Byers et al., 1987), rabbit polyclonal anti-phospho-histone H3 (1:1000, Upstate Biotechnology), mouse monoclonal anti-Hts 1B1 (1:5, DSHB), mouse monoclonal anti-Armadillo N27A1 (1:100, DSHB) and mouse monoclonal anti-Eyes Absent eya10H6 (1:200, DSHB). The fluorescence-conjugated secondary antibodies were purchased either from Molecular Probes or Jackson Immunoresearch and used at a 1:200 dilution. All samples were mounted in Cytifluor (Kent). For DNA labeling, the ovaries were additionally incubated either for 20 minutes with 0.4 mg/ml RNase A and were subsequently stained with

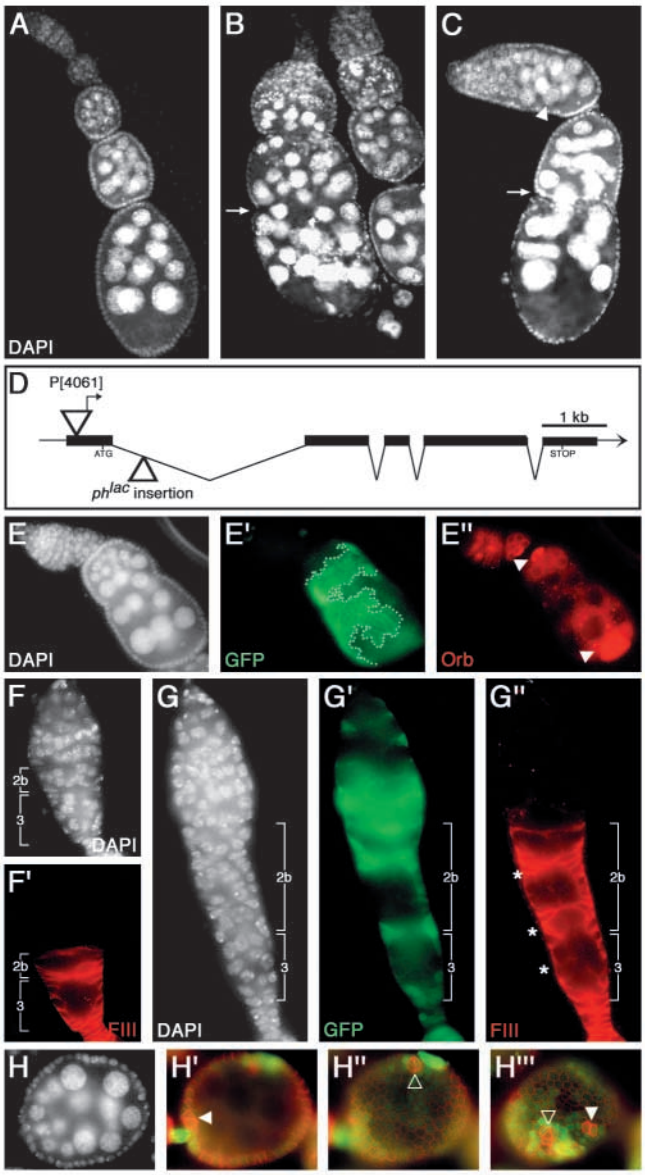

Fig. 2. Overexpression of *ph* leads to defects in follicle formation. All ovarioles are oriented with the anterior towards the top left corner. (A,B,C) DAPI staining of *4061/+* (A) and *4061/+; da-Gal4/+* (B,C) ovarioles. (B) Compound follicle with a 'curved' aspect to the follicular epithelium (arrow). (C) The first cysts in the germarium are contained within a single epithelium, without follicle individualization (arrowhead); arrow points to apposed follicles. (D) Representation of *P{y+}UAS* and *PlacW* insertions at the *ph* locus in the *P[4061]* and ph^{lac} lines, respectively. The *P{y+}UAS* is inserted 99 pb upstream of the ATG, in the sense orientation, whereas the *PlacW* is inserted in the first intron. (E-H) Control (F) and mosaic ovarioles (E,G,H) in which *ph* overexpressing cellular clones are detected by the expression of a GFP reporter construct (E′,G′,H′-H‴). Ovarioles are stained with DAPI (E,F,G,H), anti-Orb antibodies (E″) and anti-Fas III antibodies (F′,G″,H′-H‴). Compound follicle (E), with a 'curved' epithelium, results from encapsulation of two oocytes as visualized by Orb staining (E″, arrowheads). (G-G‴) Abnormal accumulation of germline cysts in an entirely clonal germarium (compare G″ and F′); prefollicular cells that overexpressed *ph* began to migrate over germline cysts (G″, asterisks) but failed to completely individualize them. (H) Compound follicle with two germline cysts encapsulated together; this follicle contains four groups of polar cells (arrowheads, H′-H‴: three different focal planes of the same follicle).

50 μg/ml propidium iodide (Molecular Probes) or with 1 μg/ml of DAPI (Sigma).

For DAPI staining, tissues were fixed in 4% formaldehyde in PBS for 25 minutes and rinsed twice, first in PBT then in PBS. Ovaries were placed for one night in PBS:glycerol (1:1), with 1 μg/ml of DAPI (Sigma). Samples were examined either with a Leica DMR microscope or by confocal microscopy using a Leica DMR-BE microscope.

Results

A gain-of-function screen reveals that somatic overexpression of *ph* results in follicle formation defects

We performed a genetic screen to identify genes that when overexpressed in the ovarian somatic line would affect early phases of oogenesis (GAL4/UAS system; see Materials and methods and Fig. 1). Female progeny from the cross between the P{y+}UAS insertion line 4061 and the *da-GAL4* line exhibited reduced fecundity compared with control *w; da-GAL4/+* and *4061/w* females (data not shown). Twenty-two percent of *4061/w; da-GAL4/+* ovarioles (*n*=202) exhibited defects in oogenesis (Fig. 2B,C), not present in control ovarioles (Fig. 2A), which concerned both germarial and vitellarial regions. We observed both compound follicles with more than 16 germline cells (Fig. 2B, arrow) and apposed follicles separated by two follicular epithelia but presenting no intervening interfollicular stalk (Fig. 2C, arrow). In these same ovarioles, germarial region 2b/3 was often abnormally swollen and long and contained advanced cysts normally corresponding to stage 5 follicles of the vitellarium (Fig. 2C, arrowhead), indicating that follicle budding is defective.

The P{y+}UAS insertion in the 4061 line was found to be located in the first exon of *ph-p*, 99 bp upstream of the ATG (Fig. 2D), in the sense orientation. This configuration, along with the fact that induction of ovarian phenotypes depends on the presence of the *da-Gal4* driver, suggests that the observed phenotypes are very likely due to an upregulation of this gene. This was substantiated by the fact that *4061/w; da-GAL4/+* females showed a partial homeotic haltere-to-wing transformation and abnormal expression of the $vestigial^{QE}$-lacZ transgene in haltere imaginal discs (data not shown) (Kim et al., 1996; Weatherbee et al., 1998). Indeed, the opposite homeotic transformation, from wing to haltere, is obtained in flies carrying homozygous/hemizygous loss-of-function *ph* alleles, which corresponds to the phenotype associated with dominant gain-of-function mutations in genes of the Antennapedia and bithorax complexes (Dura et al., 1985).

In order to confirm that somatic overexpression of *ph* perturbs oogenesis, we took advantage of available P[UAST:cDNA-*ph*] lines (*PH-C7*, *m20* and *m35*; see Materials and methods). All the *da-*, *ptc-*, *e22c-* and *hs-GAL4/UAS-ph* combinations we tested were lethal before adult eclosion. We thus used the heat-shock inducible flip-out/GAL4 system to generate mosaic follicles containing overexpressing *ph* somatic cell clones. As previously observed in *4061/w; da-GAL4/+* females, we found both compound (Fig. 2E) and apposed

follicles (data not shown), which were associated with the presence of large clones marked by GFP protein expression (Fig. 2E′). As visualized after anti-Orb staining (Orb protein accumulates specifically in the oocyte; Lantz et al., 1992), compound follicles contained two germline cysts developing together within a single follicle (Fig. 2E″, arrowheads). We next looked earlier for anomalies during germline cyst encapsulation in the germarium. In a wild-type germarium, prefollicular cells in region 2b (see Fig. 1) extend long and thin processes, which separate individual linearly arranged germline cysts. These cell extensions accumulate several cytoskeletal proteins, including Fasciclin III (Fas III) (Fig. 2F′), which is a homophilic cell adhesion molecule (Ruohola et al., 1991). When *ph* was overexpressed, germaria appeared long with multiple mature germline cysts in an extended region 2b/3 (Fig. 2G-G″, abnormal germarium in which most of the germarial cells overexpressed *ph*; Fig. 2G, same scale as Fig. 2F). These nascent follicles were only partially individualized by somatic cells, as visualized by anti-Fas III staining (Fig. 2G″, asterisks) and budding off from the germarium was severely delayed or deficient. This partial individualization of the germline cysts by prefollicular cells could explain the 'curved' aspect of the follicular epithelium systematically observed in multicyst follicles (Fig. 2B,E). As it has been shown that the absence of polar cell specification leads to formation of multicyst follicles (Grammont and Irvine, 2001), we next looked for the distribution of polar cells in multicyst follicles associated with overexpression of *ph*. We always found four groups of polar cells when two germline cysts were encapsulated together (Fig. 2H′,H″,H‴ are three different focal planes of the same multicyst follicle). In this example, two pairs of polar cells are present at each pole of the follicle (Fig. 2H′,H‴, solid arrowheads), and two groups of polar cells in the middle of the follicular epithelium (Fig. 2H″,H‴, empty arrowheads). Thus, *ph* overexpression affects the behavior of somatic germarial cells, leading to encapsulation of multiple germline cysts and delayed budding, but polar cell specification is not altered.

ph is required specifically in somatic cells for follicle formation

In order to assess the endogenous requirement of *ph*, we next looked for ovarian defects associated with induction of mitotic cell clones using the ph^{504} (noted ph^{0}) amorphic allele, which carries lesions in both *ph-p* and *ph-d* units (Dura et al., 1987). Since it has been previously reported that *ph* is transcribed and translated in both somatic and germline cells, in the germarium and in the vitellarium until stage 10, except in the oocyte (DeCamillis and Brock, 1994), we aimed at determining whether *ph* function is required in both cellular compartments, or restricted to one of them. Therefore, we first generated *ph* mutant germline clones using the Flp/FRT system and the *ovo* promoter to drive *flipase* expression specifically in the germline. Among more than 150 ovarioles with ph^{0} germline clones observed, we never detected any ovarian defects (data not shown), showing that *ph* is not required in the germline for early oogenesis.

We next generated amorphic ph^{0} somatic clones using a heat-shock promoter to drive *flipase* expression. *flipase* expression was induced at eclosion and 4-, 6- and 8-day-old females were dissected in order to recover clones in the follicular epithelium of different stage follicles. Surprisingly, no or few ph^{0} cells, detected by lack of *–lacZ* or *–GFP* reporter expression, were observed in the great majority of dissected ovarioles, while large twin clones and wild-type clones generated in parallel were readily observed (data not shown). Indeed, ph^{0} cell clones being expulsed from the somatic epithelium were frequently observed (data not shown), which probably explains the low number of clones recovered. Similar observations were reported for *ph* null clones induced in wing imaginal discs (Santamaria et al., 1989). In addition, it is possible that the division rate of ph^{0} cells may be affected.

Despite the presence of very few ph^{0} cells (Fig. 3A′), follicles with more than 16 germline cells were observed (Fig. 3A). These follicles result from the encapsulation of several germline cysts together as a constant 15:1 ratio between nurse cells and oocyte was always observed using anti-Orb staining (Fig. 3A″). Apposed follicles were also observed (data not shown). In order to explain these encapsulation defects, we examined prefollicular cell behavior in the germarium. In the germaria recovered bearing both ph^{+} and ph^{0} cells in region 2b/3 (Fig. 3B, ph^{0} cells marked by an asterisk in inset), staining with Hu-li tai shao (Hts) antibodies (Hts is present on lateral membranes of prefollicular cells; Lin et al., 1994) showed that while ph^{+} prefollicular cells had encapsulated germline cysts by extending long, thin cell processes centripetally (Fig. 3B′, arrow), ph^{0} cells remained at the periphery of the germarium with a relatively round morphology in comparison (Fig. 3B′, asterisks in inset). These results suggest that *ph* function is necessary in prefollicular cells for these cells to be able to acquire their specific adhesive and/or migratory properties necessary for proper encapsulation of individual germline cysts.

As early aspects of the prefollicular cell developmental program are affected in ph^{0} cells, we next asked whether their subsequent differentiation into interfollicular, polar and follicular cells was also affected. Whereas wild-type interfollicular stalks contain five to seven cells organized in a single line, we noted the presence of abnormal stalks, with more than a dozen cells organized in two or three lines upon induction of ph^{0} mutant cell clones (Fig. 3C-C″). In some cases, these abnormal stalks were mosaic, containing both ph^{0} and ph^{+} cells (Fig. 3C′), although the majority recovered were made up exclusively of ph^{+} cells.

We next examined expression of polar cell markers. In wild-type follicles, polar cells are arranged as two groups of somatic cells at each follicle extremity, as detected by various markers including Eyes Absent (Fig. 3E″, arrowheads), Fas III (see Fig. 5A″, arrow) and several enhancer trap lines (such as A101, see Fig. 5F′, inset). It has been previously described that in wild-type females the number of cells expressing these markers is greater than two at the extremities of young follicles (in particular stage 2 and 3 follicles), then progressively restricted to two by stage 5 (Besse and Pret, 2003). The Eyes Absent (Eya) protein is normally expressed in prefollicular cells then specifically turned off in polar cells, while maintained in follicular cells (Bai and Montell, 2002; Torres et al., 2003) (Fig. 3E″, arrowheads). When ph^{0} clones were induced, two groups of anterior and posterior polar cells were invariantly observed both in normal and multicyst follicles (data not shown). However, the presence of more than two polar cells at the extremities of stage 5 or older follicles was observed in rare

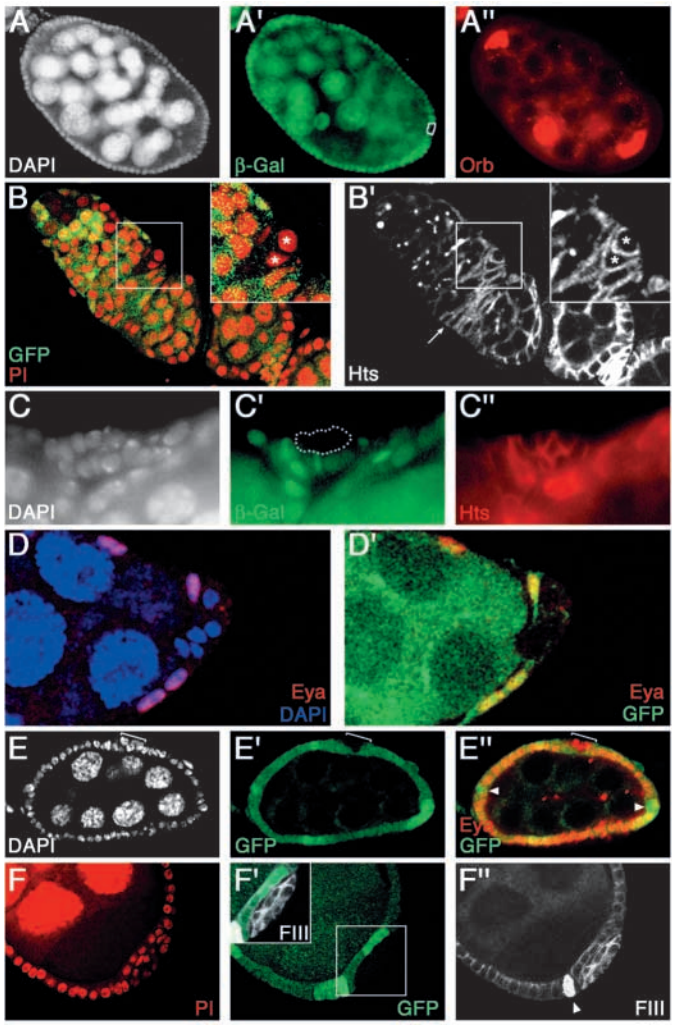

Fig. 3. *polyhomeotic* is required specifically within somatic cells to control somatic cell differentiation. (A-F) ph^0 mosaic follicles stained with DAPI (A,C,E, gray; D, blue), Propidium Iodide (B,F, red), anti-β-Gal (A′,C′, green), anti-Orb (A″, red), anti-Hts (B′, white; C″, red), anti-Eya (D,D′,E″, red), or anti-Fas III (F′, insert and F″, white) antibodies. Mutant clones for ph^0 are detected by the absence of anti-β-Gal antibody staining (A′,C′, white tracing) or by the absence of GFP fluorescence (B,D′,E′,F′, green). (A-A″) Mosaic compound follicle (A), bearing a small ph^0 mutant somatic cell clone (A′) and exhibiting three oocytes (A″). (B,B′) ph^0 prefollicular cells (asterisks in insets), in contrast to wild-type cells (B′, arrow), are found at the periphery and do not extend thin processes over germline cysts. (C-C″) Abnormal mosaic ph^0 and ph^+ interfollicular stalk. Hts accumulates at the membrane of interfollicular stalk cells (C″). (D-D′) In a late stage follicle, a clone of ph^0 cells exhibiting five polar cells (D,D′, marked by the absence of anti-Eya staining). (E-E″) ph^0 cells (E′) expulsed from the follicular epithelium (E, bracket) express the Eya protein (E″). Arrowheads in E″ point to pairs of polar cells at each follicle extremity that do not express Eya. (F-F″) ph^0 follicular clones (F′, insert) upregulate Fas III compared with adjacent ph^+ follicular cells (F″), but nonetheless express lower levels of Fas III compared with polar cells (F″, arrowhead). ph^0 clones are delimited by a dotted line in A′ and C′.

ph^0 polar cell clusters, marked by the absence of Eya expression, indicating an excess compared to wild-type follicles at these stages (Fig. 3D,D′) (five polar cells do not express Eya). Importantly, ph^0 follicular cells correctly express Eya protein (Fig. 3E-E″, bracket), like neighboring ph^+ follicular cells (Fig. 3E-E″).

Finally, we observed that ph^0 follicular cells expressed Fas III at a higher level than neighboring ph^0/ph^+ or ph^+/ph^+ follicular cells (Fig. 3F′, insert, and F″). A high level of Fas III is characteristic of both polar and undifferentiated prefollicular cells. Since the level of Fas III expressed by ph^0 cells is lower than that expressed by neighboring polar cells (Fig. 3F″, arrowhead), it is possible to conclude that these ph^0 cells represent undifferentiated follicular cells. Thus, induction of ph^0 somatic cell clones in the ovary perturbs germline cyst encapsulation and stalk morphogenesis, generates an excess of stalk and polar cells and maintains follicular epithelial cells in a precursor state.

ph mutant germaria exhibit abnormal encapsulation by prefollicular cells and abnormal budding of germline cysts

We next examined ovaries associated with a whole-fly *ph* partial loss-of-function context. We found ovarian defects associated with one of the previously described homozygous viable ph^{lac} mutations (Dura et al., 1987) (Fig. 2D) and showed that these defects are specifically due to this mutation, since they are maintained after outcrossing over six generations (data not shown). As visualized after DAPI staining, ph^{lac} ovarioles exhibited follicles with either more (Fig. 4A,B; compare to wild-type ovariole in Fig. 2A) or fewer (Fig. 4C, arrow) than 16 germ cells. When a follicle contained more than 16 germ cells (23% ovarioles contained at least one compound follicle over 248 ovarioles observed), we found groups of nurse cells with either equivalent (Fig. 4A, arrow) or different (Fig. 4B, arrows) degrees of polyploidy. All compound follicles were multicyst, since they contained several oocytes (anti-Orb staining, data not shown). In addition, apposed chambers were also observed in ph^{lac} females (data not shown). When a follicle contained fewer than 15 nurse cells (5.6%, n=248), one of the adjacent chambers contained the complementary number of nurse cells or degenerating nurse cells (Fig. 4C; empty arrowhead points to a pycnotic nucleus). Thus, perturbing *ph* function in the ph^{lac} mutant leads to defects in follicle formation.

In the ph^{lac} mutant, encapsulation defects were correlated to abnormal behavior of prefollicular cells. In 14.5% (n=248) of the germaria, delayed budding of the emerging follicles was found (Fig. 4D-G). In these germaria, germline cyst encapsulation was defective in different ways. Whereas germline cysts arrange themselves in a linear fashion in region 2b of wild-type germaria (Fig. 2F′), numerous cysts were found encapsulated in an anarchic fashion in ph^{lac} mutant germaria (Fig. 4D′, arrowheads). Associated with this, groups of Fas III$^+$ prefollicular cells were found aggregated at the periphery of the germarium not participating in the encapsulation process (Fig. 4D′,G′, asterisks). In other germaria, abnormal prefollicular cell encapsulation leads to splitting of germline cysts and envelopment of fewer than 16 germline cells (Fig. 4E″). In more strongly affected ovarioles, an absence of prefollicular cell migration between adjacent germline cysts was associated with an accumulation of several mature cysts in region 3 of the germarium (Fig. 4F′,F″, arrows). In these cases, prefollicular cell invagination was observed anteriorly (Fig. 4F′,F″,

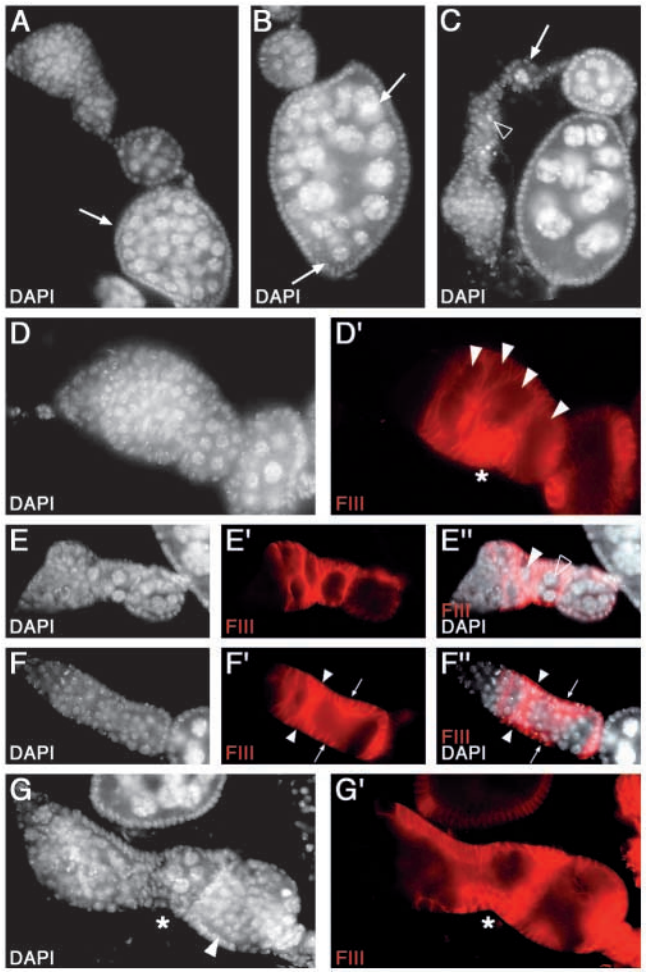

Fig. 4. *ph*^{lac} mutant germaria exhibit abnormal encapsulation by prefollicular cells and abnormal budding of germline cysts. (A-G) DAPI staining (A-G,E″,F″) and anti-Fas III staining (D′,E′,E″,F′,F″,G′) of *ph*^{lac} mutant ovarioles. (A,B) arrows point to compound follicles, with the same degree (A), or different degree (B) of polyploidy of the nurse cells. (C) *ph*^{lac} follicle with fewer than 16 nurse cells (one nurse cell, arrow). No complementary chamber could be found, probably due to nurse cell degeneration (empty arrowhead points to pycnotic nucleus). (D-G) In *ph*^{lac} germaria, cysts are haphazardly distributed (D′, arrowheads), and prefollicular cells fail to migrate, remaining at the periphery (D′,G′, asterisks). Encapsulation defects include splitting of germline cysts (E′,E″, arrowhead and empty arrowhead point to follicles with fewer than 15 nurse cells, having the same degree of polyploidy as the last egg chamber in germarial region 3) or to several cysts encapsulated together (F′,F″; arrows point to an absence of prefollicular cell migration between adjacent germline cysts, whereas arrowheads point to an invagination of prefollicular cells). (G) In strongly affected ovarioles, prefollicular cells completely failed to migrate (G′, asterisks), leading to the inclusion of several germline cysts in the same follicle. Arrowhead points to a 'highly multicystic' follicle.

arrowheads) which will therefore probably lead to budding of a multicyst follicle. In other abnormal ovarioles, no clear limit between germarium and vitellarium could be found; in these cases, Fas III expression remained very strong all along the ovariole (Fig. 4G′). Thus, as described above for *ph*^{0} cells, we also observed an alteration in the differentiation of *ph*^{lac} prefollicular cells impaired in their adhesive and migratory properties during germline cyst encapsulation.

Differentiation of interfollicular and polar cells is affected in *ph*^{lac} mutants

As differentiation of specialized germarial somatic cells are affected in *ph*^{0} clones, we next examined expression of stalk and polar cell differentiation markers in *ph*^{lac} females. In wild-type ovarioles, interfollicular stalk cells express high levels of α-Spectrin (Fig. 5A′, inset), and low levels of Fas III (Fig. 5A″, inset). We found that *ph*^{lac} mutant stalks contained an excess number of cells (10 to 50) frequently organized into two or three lines (32%, *n*=231) (Fig. 5B,C). Such structures were comprised of cells with at least some characteristics of interfollicular stalk cells because they strongly expressed α-Spectrin (Fig. 5B′). However, since they also strongly express Fas III (Fig. 5C′), their differentiation state was ambiguous.

Second, we examined polar cell differentiation. In wild-type follicles, polar cells are detected using either anti-Fas III antibody or the enhancer trap line A101 (*neuralized*-lacZ), which are both markers for mature polar cells from stage 2 onward (data not shown and Fig. 5A″, arrow). In *ph*^{lac} ovarioles, whether follicles contained more or fewer than the normal number of germline cells, there were always two groups of polar cells, one at each extremity, anterior and posterior (Fig. 5D,D′, follicle with fewer than 15 nurse cells; Fig. 5E,E′, multicyst follicle). However, these groups often contained more than two polar cells (Fig. 5F,F′, insets; 11 polar cells can be seen at the posterior pole of a stage 4 multicyst follicle). Although it has been shown previously that early stage 2 and 3 follicles in wild-type ovarioles also frequently contain more than two polar-cell marker expressing cells, a maximum of five of these cells was observed (Besse and Pret, 2003) (Fig. 5G). In addition, by stage 4, in wild-type ovarioles, the restriction to two polar cells at each pole was almost complete and from stage 5 onwards there were never more than two observed (Besse and Pret, 2003) (Fig. 5G). In contrast, *ph*^{lac} mutant ovarioles exhibited a significant proportion of stage 4, as well as some stage 5 and up to stage 9, follicles with more than two polar cells (Fig. 5G and data not shown). The presence of groups with more than two polar cells after stage 4 was not strictly correlated with an abnormal number of germline cells within the follicle but was always associated with an abnormal interfollicular stalk. However, abnormal interfollicular stalks associated with two polar cells at each extremity were also observed. In addition, *ph* mutant ovarioles exhibited a shift in the dynamics of A101 staining: the appearance of this staining is delayed since 100% (*n*=112) of stage 2 *ph*^{lac} follicles exhibited no marked cells compared to 34% (*n*=78) of stage 2 wild-type follicles. In conclusion, *ph* mutations perturb interfollicular stalk cell differentiation and stalk morphogenesis, delay the polar cell differentiation program and generate an excess of both of these cell types. Since stalk and polar cells arise from the same precursors (Tworoger et al., 1999), the differentiation of these precursors seems specifically affected in *ph*^{lac} mutants.

Proliferation of germarial somatic cells is reduced in *ph*^{lac} mutant females

We next wanted to know whether the excess in number of polar and interfollicular stalk cells was associated with an

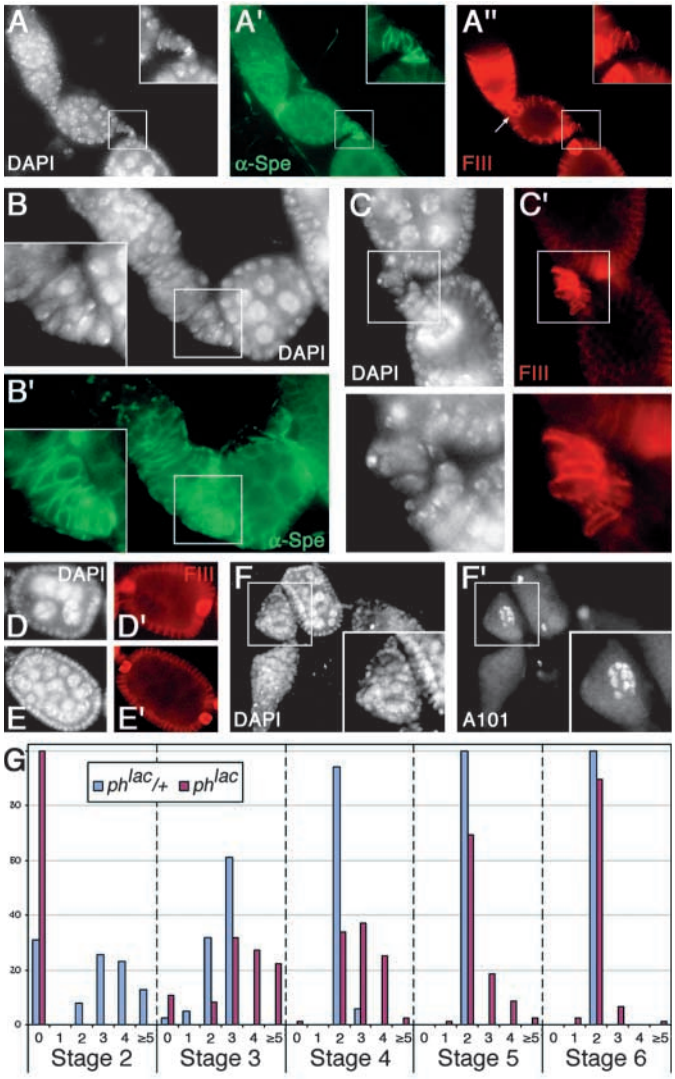

Fig. 5. *polyhomeotic* function is required for interfollicular stalk and polar cell differentiation. (A-C) Nuclear DAPI staining reveals the presence of long stalks in ph^{lac} mutant ovarioles (B,C, compare to wild-type, A). (A) In wild-type interfollicular stalk cells, α-Spectrin (α-Spe) is strongly expressed (A′, inset), whereas Fas III (FIII) is almost absent (A″, inset). (B,C) In ph^{lac} ovarioles, long interfollicular stalks fail to differentiate correctly as interfollicular stalk cells express both α-Spe (B′, inset) and Fas III (C′, inset). (D,E) ph^{lac} follicles with fewer (D) or more (E) than 16 germline cells, containing one pair of polar cells at each pole, as revealed after staining with anti-Fas III antibody (D′,E′). (F,F′) DAPI staining (F) and anti-β-Gal antibody staining (F′) of a compound stage 4 follicle in a ph^{lac}/ph^{lac};*A101/+* ovary. (G) Percentage of ph^{lac}/+; *A101/+* (blue) and ph^{lac}/ph^{lac}; *A101/+* (pink) stage 2 to 6 egg chambers containing 0-5 and more polar cells ($n \geq 34$ for ph^{lac}/+; *A101/+* and $n \geq 56$ for ph^{lac}/ph^{lac}; *A101/+*for each stage). These data concern anterior polar cells.

alteration in proliferation of precursor cells. We investigated the proliferative activity of ph^{lac} germarial somatic cells using antibodies against phospho-histone 3 (PH3), which specifically stain cells undergoing mitosis (Hendzel et al., 1997), and anti-Fas III antibodies to identify the different regions of the germarium (e.g. Zhang and Kalderon, 2001)

Table 1. Number of mitotic somatic cells per ovariole

Ovarioles	Stem/inner sheath cells	Region 2b	Region 3
ph^{lac}/w^{1118}	0.10	0.28	0.52
ph^{lac}	0.06*	0.16†	0.34‡

Each number is the mean from 225 or more ovarioles observed.
*P=0.2, †P=0.01 and ‡P=0.002 in comparison with wild type.

(Fig. 2F′). As no positive marker for ovarian somatic stem cells has been described so far, and as inner germarial sheath cells in 2a/2b junction have been recently shown to be mitotically active (Song and Xie, 2002; Song and Xie, 2003), any $PH3^+$ somatic cells found just anteriorly to Fas III staining were considered to be somatic stem cells and inner sheath cells. Control ph^{lac}/+ and mutant ph^{lac} ovarioles were double-stained with antibodies against Fas III and PH3, and the number of proliferating stem and inner sheath cells (between region 2a and 2b), prefollicular cells (region 2b), and follicular cells (region 3) was scored for each genetic background. Very abnormal ovarioles, as those shown in Fig. 4G′, were not included in this study because the different germarial regions could not be delimited easily. As shown in Table 1, the number of mitotic somatic cells is significantly lower for ph^{lac} females compared to ph^{lac}/+ control sisters for prefollicular and follicular cells in regions 2b and 3 of the germarium. Although there is also a difference between these two genetic contexts for mitotic activity in the 2a/2b region, the low frequency of this type of event and the presence of inner sheath cell mitotic activity may explain the fact that it is not possible to demonstrate the statistical significance of this difference. We also verified that, as in the wild-type context, there is no proliferation of stalk and polar cells after stage 1 in ph^{lac} ovarioles (data not shown). Therefore, the excess number of stalk and polar cells observed in *ph* mutant ovarioles cannot be explained by excess proliferation of precursors in the germarium. Nonetheless, the reduced proliferation of prefollicular and follicular cells in the germaria of *ph* mutant ovarioles probably contributes to the germline cyst encapsulation and budding defects observed.

Other mutations in *Pc*-G genes lead to ovarian defects

As this is the first time that a study reveals a role for a *Pc*-G member in ovarian folliculogenesis, we next analyzed the role of other members of the *Pc*-G for early oogenesis defects by clonal analysis using amorphic mutations. We first analyzed mutant Sce^1 somatic mitotic clones. The viability of Sce^1 cells does not seem to be affected since Sce^1 cell clones are produced at a high frequency and since these clones have the same size as twin clones (Fig. 6A′ and data not shown). Associated with induction of Sce^1 cell clones, we observed several defects, including multicyst follicles with several oocytes (Fig. 6A; two oocytes are indicated by arrows) and disorganized encapsulation in germarial region 3 (Fig. 6B,B′). In addition, abnormally long interfollicular stalks containing from 10 to over 50 cells were found between wild-type (Fig. 6C) or abnormal follicles (Fig. 6B, arrowhead). In some cases, a continuous cable of somatic cells can be observed lying along one side of the follicles (Fig. 6D). These defects were always

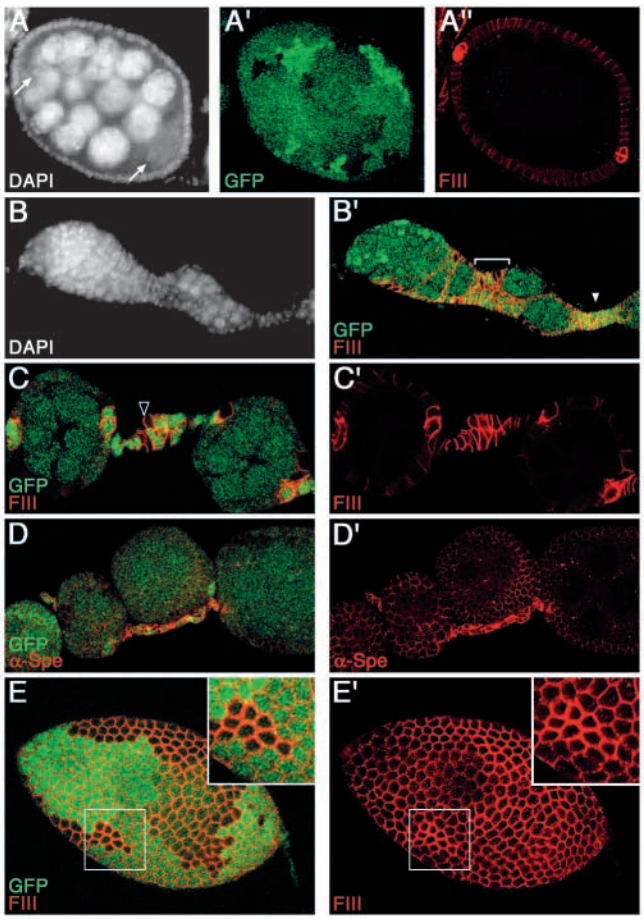

Fig. 6. Mutations in the *Sce*^{1} gene lead to ovarian defects. (A-E) *Sce*^{1} mosaic follicles stained with DAPI (A,B), anti-Fas III (A″,B′,C,C′,E,E′), or anti-α-Spectrin (D,D′) antibodies. Mutant clones for *Sce*^{1} are detected by the absence of GFP (A′,B′,C,D,E, green). (A-A″) Compound follicle (A, arrows point to oocyte nuclei) bearing several *Sce*^{1} clones marked by the absence of GFP staining (A′) and exhibiting two pairs of polar cells at the anterior and posterior poles (A″). (B-B´) Mosaic *Sce*^{1}/*Sce*$^{+}$ germarium. *Sce*^{1} mutant prefollicular cells do not migrate correctly arround germline cysts (B′, bracket). Arrowhead points to an abnormal interfollicular stalk with *Sce*^{1} cells on another focal plane. (C,D) Interfollicular stalks are very long and all cells express Fas III (C,C′) as well as α-Spectrin (D,D′) at high levels compared with adjacent follicle cells. Empty arrowhead (C) points to one *Sce*^{1} cell (four other *Sce*^{1} cells are present in this stalk). (E-E′) Mosaic *Sce*^{1}/*Sce*$^{+}$ follicle. *Sce*^{1} follicular cells strongly express Fas III protein compared with adjacent *Sce*$^{+}$ cells.

found associated with the presence of *Sce*^{1} mutant cells, although few *Sce*^{1} cells suffice to create a very abnormal stalk (Fig. 6C, empty arrowhead). All interfollicular cells from an abnormal mosaic stalk strongly express α-Spectrin as in wild-type stalks (Fig. 6D,D′); however, abnormal perdurance of Fas III was also observed (Fig. 6C,C′). Therefore, as described for *ph* mutations, interfollicular stalk cells display an intermediate differentiation state. This was also the case for follicular cells since *Sce*^{1} mutant epithelial follicular cells also exhibited strong expression of the Fas III protein compared with neighboring *Sce*$^{+}$ cells (Fig. 6E,E′, insets). Finally, no excess of polar cells was observed when *Sce*^{1} clones were induced in these cells (data not shown).

Almost indistinguishable defects (multicyst follicles and giant stalks) were also obtained with the *Scm*^{D1} mutation when mutant clones were induced under the same conditions (data not shown). In contrast, no ovarian defects were provoked upon induction of somatic mitotic clones using amorphic mutant alleles for four other *Pc*-G genes under the same conditions, namely *Psc*, *Su(z)2*, *Asx* and *Pcl*.

Discussion

Gain-of-function and loss-of-function studies reveal a new role for the *ph* gene in early oogenesis

During *Drosophila* oogenesis, a new role for *ph* was first revealed by the reduced fecundity and associated ovarian anomalies observed upon analysis of a P{y+}UAS insertion in the first exon of the *ph-p* locus (*4061* line). That the ovarian phenotypes characterized for this line are due to overexpression of *ph* is supported by three lines of evidence: (1) the ovarian phenotypes produced depend on the presence of a *GAL4* driver (*da-GAL4*); (2) the *4061/w; da-GAL4/+* flies also present a *ph* gain-of-function haltere-to-wing transformation; and (3) flip-out clone overexpression in ovarian somatic cells of several *UAS-ph* cDNA transgenes gave very similar ovarian phenotypes. In particular, overexpression of *ph* is associated with production of multicyst follicles in which several (two to four) germline cysts develop within a single follicular epithelium. Importantly, each multicyst follicle contains several pairs of polar cells corresponding to the number of cysts present in the follicle. Therefore, unlike for other mutants [Notch (Grammont and Irvine, 2001) and Hedgehog (Forbes et al., 1996)] for which inclusion of several cysts in one follicle has been attributed to a problem in polar cell specification, this does not seem to be the case here. Interestingly, multicyst follicles produced by *ph* overexpression are covered by a follicular epithelium that is not completely regular, showing indentations that appear to mark boundaries between cysts as evidenced by the presence of polar cells at the level of the indentations. This suggests that, earlier, cyst individualization may have begun and been subsequently aborted. In support of this, analysis of the associated germaria showed an abnormally long region 3, with adjacent mature germline cysts between which prefollicular cells fail to complete centripetal migration (visualized by specific anti-Fas III antibody staining of prefollicular cells). Overexpression of *ph* may thus specifically affect the expression of proteins necessary for recognition and/or adhesion between prefollicular cells and germline cysts for encapsulation. These effects seem to be specific to this stage since later interactions between these two cell lineages, for instance between follicular epithelial cells and the nurse cells and oocyte, are not perturbed by overexpression of *ph*.

Surprisingly, a similar phenotype to that observed in the *ph* overexpression study, multicyst follicles (several cysts within one follicle), was observed with loss-of-function *ph* alleles (discussed further below). Importantly, in contrast to *ph* overexpression, multicyst follicles in *ph* loss-of-function mutant ovaries always have only two groups of polar cells, one at each pole. Therefore, it seems that, unlike for overexpression of *ph*, delayed or deficient polar cell specification in *ph* mutants

contributes to inclusion of several cysts within a single follicle. Thus, *ph* overexpression and loss-of-function phenotypes are distinguishable, indicating that the origin of the phenotypes is probably different.

ph function is necessary in somatic cells of the germarium for both their proliferation and differentiation

The implication of the *ph* gene in ovarian somatic cells was also studied using two different loss-of-function mutations: the hypomorphic ph^{lac} mutation, which consists of a *PlacW* transposon inserted in the first intron of *ph-p* (Fauvarque et al., 1995); and via clonal analysis of the amorphic ph^{504} (noted ph^{0}) allele, which eliminates the functions of both *ph-p* and *ph-d* (Dura et al., 1987). The origin of the multicyst phenotype caused by *ph* loss-of-function mutations was characterized more precisely by analysis of the process of follicle formation in the germarium. This study showed that several early aspects of the somatic cell developmental program (including proliferation, morphogenesis and differentiation) are perturbed by these *ph* mutations.

On the one hand, the rate of division of germarial somatic cells is reduced in a *ph* hypomorphic mutant background as assayed by immunohistochemical analysis of the mitosis-specific PH3. This probably contributes to delayed follicle encapsulation and budding, evidenced by the accumulation of mature germline cysts in germarial region 3 of *ph* mutant ovarioles, and, consequently, by the formation of multicyst follicles. Although the same type of analysis was not possible upon induction of clones of the ph^{0} amorphic mutation in somatic ovarian cells, the fact that these clones are very small or absent compared with control clones suggests that a proliferation defect may also be associated with this *ph* mutation.

On the other hand, the morphogenetic properties of prefollicular cells and their differentiation into polar cells, interfollicular stalks and follicular epithelia are also specifically perturbed in *ph* mutants, which also probably contributes to formation of multicyst follicles. Our ph^{0} clonal analysis showed that *ph* function is necessary specifically in somatic cells, and not in the germline, for proper follicle formation. Almost all the phenotypes observed in *ph* mutant ovaries were reproduced upon induction of ph^{0} clones in prefollicular cells and their descendants.

First, prefollicular cell individualization of germline cysts is compromised in germarial regions 2a and 2b of *ph* mutant ovarioles. Fas III, which is specifically upregulated in prefollicular cells in wild-type germaria, is expressed normally in *ph* mutant prefollicular cells (ph^{lac} and ph^{0}), but these cells remain at the periphery of the germarium and fail to undergo normal morphological changes necessary for germline cyst encapsulation, allowing multiple cysts, not individualized by somatic cells, to accumulate in region 3. In other *ph* mutant germaria in which prefollicular cells have migrated between germline cysts, encapsulation is disorganized, and germline cysts can be split and follicle budding significantly delayed. Therefore, in the germarium, interaction between prefollicular cells and the germline is defective.

Second, prefollicular cell differentiation into polar cells, interfollicular stalk cells and follicular epithelial cells is delayed and/or incomplete in the presence of *ph* mutations. Using a polar cell specific marker (A101/*neu-lacZ*), we showed that specification of polar cells, normally appearing by stage 2 in wild-type follicles, was delayed in *ph* mutant ovarioles until stage 3. In addition, multicyst follicles produced in *ph* mutant ovarioles contained two pairs of polar cells, one at each extremity of the anterior/posterior axis. Therefore, polar cell specification, which is necessary for individualization of germline cysts, is perturbed by *ph* mutations. Interfollicular stalk cell differentiation was assayed by expression of the stalk-specific marker, α-Spectrin, and by loss of Fas III, which is normally expressed at high levels in precursor prefollicular cells. In stalks of both *ph* mutant and ph^{0}/ph^{+} mosaic ovarioles, although α-Spectrin expression was normally upregulated, Fas III was also present at high levels, indicating an ambiguous state of differentiation. Poor differentiation of stalk cells was further substantiated by the abnormally long and disorganized interfollicular stalks that showed intercalation defects. Abnormal perdurance of the early prefollicular cell marker Fas III was also observed at the level of the follicular epithelium in very affected *ph* mutant ovarioles, as well as in ph^{0} epithelial cell clones. Taken together, these results suggest that *ph* mutations result in the prolongation of a precursor state for polar, stalk and follicular epithelial cells.

Third, *ph* mutant ovarioles exhibit an excess of polar cells (up to 11) at both the anterior and posterior poles of follicles, which persists beyond stage 9, accompanied by an excess of adjacent interfollicular stalk cells (from 10 to 50). ph^{0} clones induced in the polar cell lineage also produced an excess of polar cells after stage 5, and the presence of ph^{0} cells was also associated with abnormally long stalks. Overproliferation of the pool of precursor cells common to both polar and stalk cells would result in an excess of these cells. However, the mitotic activity of germarial somatic cells was assayed by PH3 staining and a reduction compared to wild-type was observed in regions 2a, 2b and 3. It has recently been shown that an excess of polar cells is normally present in early stage follicles in wild-type females, and that the final pair of polar cells is selected from this group via apoptosis-induced cell death (Besse and Pret, 2003). However, in this study, a maximum of six such pre-polar cells was observed when apoptosis was specifically blocked. Since *ph* mutant ovarioles exhibit up to 11 polar cells, it would seem that apoptosis of pre-polar cells is probably not the only aspect of polar cell development affected. Finally, it is possible that the process by which the pool of polar and stalk cell precursors, distinct from the progenitors of the epithelial follicle cells, is set aside may be affected by *ph* mutations, leading to both problems in their number and differentiation (Tworoger et al., 1999). Determination of this pool probably involves several cell-cell signaling pathways in region 2b of the germarium, implicating Delta/Notch and EGFR signaling initiating from the germline and Hedgehog signaling from anterior terminal filament somatic cells (Goode et al., 1996b; Zhang and Kalderon, 2000; Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001; Besse et al., 2002). *ph* may participate, in parallel or within one (or several) of these signaling pathways, to the regulation of the somatic cell differentiation program in the germarium. So far, attempts to uncover genetic or molecular interactions between *ph* and genes of these signaling pathways have proven unfruitful (K.N., unpublished results).

Requirements for the Polycomb group in oogenesis

The *ph* gene was first characterized as one of the *Drosophila Pc*-G genes, which encode transcriptional repressors required for maintaining the spatial pattern of homeotic gene expression during embryonic and larval development (Christen and Bienz, 1994; McKeon et al., 1994; Soto et al., 1995). *ph* has additional functions during development, since it has also been implicated in restriction of anterior compartment expression of *engrailed* and *hedgehog* in the wing imaginal disc (Maschat et al., 1998; Maurange and Paro, 2002). Our present results, which implicate *ph* function and that of two other *Pc*-G genes (*Sce* and *Scm*) in somatic cell development during early oogenesis, thus suggest that *Pc*-G function may be more generalized than previously thought. One other study reported ovarian defects associated with two temperature-sensitive alleles ($pco^{ox736hs}$ and $pco^{my939hs}$) of the *E(z)* gene, but the defects observed do not resemble those of *ph* (degeneration of nurse cells and little growth in the size of the follicle beyond stage 3 or 4) (Philips and Shearn, 1990).

We also examined ovarian phenotypes associated with mutations in several other *Pc*-G genes. The product of the *Scm* gene interacts directly with PH (Peterson et al., 1997), and, with PH, forms part of the same complex, PRC1, in *Drosophila* embryos (Shao et al., 1999). We found that the presence of large somatic cell clones of Scm^{D1} (amorphic mutation) leads to similar, though not completely overlapping, phenotypes than those observed for mutations in the *ph* gene. In particular, our results suggest that somatic cells are poorly differentiated and this leads to formation of multicyst follicles and abnormal interfollicular stalks with an excess number of cells. We also tested other *Pc*-G members, some which also belong to the PRC1 complex (*Sce* and *Psc*), while others do not (*Asx*, *Su(z)2* and *Pcl*). Ovarian defects were observed only with a mutation in the *Sce* gene, and these defects closely resembled those obtained with Scm^{D1}. Since follicle cell clones mutant for Scm^{D1} and Sce^{1} covered large areas of the follicular epithelium, like wild-type clones, we can conclude that, unlike for ph^{0}, Scm^{D1} and Sce^{1} somatic cells are not affected in their proliferative property and/or viability. In addition, Scm^{D1} and Sce^{1} mutations did not affect polar cell number or differentiation. These results indicate that these anomalies were specific to mutations in the *ph* gene. Thus, we show that several components of the PRC1 complex, but not all, seem to be implicated in follicle formation and their functions do not seem to overlap fully. In addition, none of the genetic interactions between *Pc-G* genes known to exist for embryonic segment identity were reproduced in the ovary system (data not shown). We can make two hypotheses concerning the role of these *Pc-G* genes in ovarian folliculogenesis: (1) either each *Pc-G* gene acts specifically on its own specific subset of target genes in somatic cells of the ovary, possibly regulating the transcriptional machinery directly rather than forming particular Pc-G complexes that alter chromatin structure; or (2) repression of target genes in somatic cells of the ovary occurs via *Pc-G* complexes in a chromatin-dependent manner, but the complexes involved differ markedly in composition from those identified for embryonic cell identity. Further experiments will be needed to distinguish between these two possibilities.

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