Multiple roles of the F-box protein Slimb in *Drosophila* egg chamber development

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Accepted 29 March 2005

Development 132, 2561-2571
Published by The Company of Biologists 2005
doi:10.1242/dev.01839

Summary

Substrate-specific degradation of proteins by the ubiquitin-proteasome pathway is a precise mechanism that controls the abundance of key cell regulators. SCF complexes are a family of E3 ubiquitin ligases that target specific proteins for destruction at the 26S-proteasome. These complexes are composed of three constant polypeptides – Skp1, Cullin1/3 and Roc1/Rbx1 – and a fourth variable adapter, the F-box protein. Slimb (Slmb) is a *Drosophila* F-Box protein that fulfills several roles in development and cell physiology. We analyzed its participation in egg chamber development and found that *slmb* is required in both the follicle cells and the germline at different stages of oogenesis. We observed that in *slmb* somatic clones, morphogenesis of the germarium and encapsulation of the cyst were altered, giving rise to egg chambers with extra germline cells and two oocytes. Furthermore, in *slmb* somatic clones, we observed ectopic Fasciclin 3 expression, suggesting a delay in follicle cell differentiation, which correlated with the occurrence of ectopic polar cells, lack of interfollicular stalks and mislocalization of the oocyte. Later in oogenesis, Slmb was required in somatic cells to specify the position, size and morphology of dorsal appendages. Mild overactivation of the Dpp pathway caused similar phenotypes that could be antagonized by simultaneous overexpression of Slmb, suggesting that Slmb might normally downregulate the Dpp pathway in follicle cells. Indeed, ectopic expression of a *dad-LacZ* enhancer trap revealed that the Dpp pathway was upregulated in *slmb* somatic clones and, consistent with this, ectopic accumulation of the co-Smad protein, Medea, was recorded. By analyzing *slmb* germline clones, we found that loss of Slmb provoked a reduction in E2f2 and Dp levels, which correlated with misregulation of mitotic cycles during cyst formation, abnormal nurse cell endoreplication and impairment of dumping of the nurse cell content into the oocyte.

Key words: *Drosophila*, Slmb, Oogenesis, Egg chamber, Eggshell

Introduction

Destruction of intracellular proteins at the 26S proteasome is a finely regulated process that determines the half-life of most key cell regulators (Hershko and Ciechanover, 1998). The proteasome is a 25 MDa multi-subunit protease complex that recognizes and degrades proteins that have been tagged with a poly-ubiquitin chain (Voges et al., 1999). The poly-ubiquitin chain is synthesized on the substrate that must be degraded by the sequential activities of three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an E3 ubiquitin ligase. It is now widely accepted that specificity of the ubiquitination process relies on the selective recognition of substrates by specific E3 enzymes (Pickart, 2001).

SCF complexes are a family of E3 ubiquitin ligases that have been reported to target different signaling molecules and cell cycle regulators (Deshaies, 1999; Zheng et al., 2002). They are composed of three relatively constant polypeptides – Skp1, Cullin1/3 and a Ring finger domain protein, Roc1/Rbx1 (Kamura et al., 1999; Ohta et al., 1999) – and one variable component – the F-box protein – that is specific for a particular substrate or small group of substrate proteins (Patton et al., 1998; Winston et al., 1999). The *Drosophila* F-box protein Slimb (Slmb) fulfills several functions in development and cell physiology: it participates in E2f destruction at the beginning of the S phase of the cell cycle (Heriche et al., 2003); it is necessary for normal circadian rythmicity by targeting the clock protein Period (Grima et al., 2002; Ko et al., 2002); it plays a role in limiting centrosome duplication (Wojcik et al., 2000) by destroying the inhibitor of the Anaphase Promoting Complex Emi1 (Margottin-Goguet et al., 2003); it represses the immune response through downregulation of the transcription factor Relish (Khush et al., 2002); and it participates in imaginal wing, limb and eye development through the modulation of Wingless, Hedgehog and Dpp/TGF-β pathways (Jiang and Struhl, 1998; Miletich and Limbourg-Bouchon, 2000; Ou et al., 2002; Theodosiou et al., 1998). In order to assess new functions of the Slmb-containing SCF complex in *Drosophila* development, we have begun to study the role of Slmb during oogenesis.

The *Drosophila* ovary is made up of 16-20 chains of egg chambers of progressive age, called ovarioles. Egg chambers are formed at the anterior end of each ovariole in a structure called germarium that harbors both germline and somatic stem cells (Margolis and Spradling, 1995; Wieschaus and Szabad, 1979). The germarium can be divided into three regions. In

region I, at the anterior part, a germline stem cell divides asymmetrically producing one daughter stem cell and one cystoblast. The cystoblast undergoes four rounds of mitosis with incomplete cytokinesis, giving rise to a cyst formed by 16 germ cells interconnected by ring canals (de Cuevas et al., 1997; Spradling, 1993). At germarium region IIa, the two germ cells with four ring canals enter meiosis and become pro-oocytes; afterwards, one of them is selected to become the oocyte while the other 15 cells develop as nurse cells. In region IIb, the cyst is contacted posteriorly and surrounded by somatically originated follicle cells (FC), thus acquiring a lens shape. Later on, in region III, the cyst re-shapes into a sphere, giving rise to an egg chamber that buds off from the germarium covered by a monolayer of FC (van Eeden and St Johnston, 1999). At this stage, the oocyte localizes to the posterior of the cyst, attaching through specific interactions to posterior FC of the egg chamber (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998).

As the egg chamber leaves the germarium, three types of FC have differentiated: stalk cells, polar cells and cuboidal FC (Dobens and Raftery, 2000; Torres et al., 2003). Stalk cells are a subtype of five to eight FC that separate adjacent egg chambers; polar cells are two pairs of FC located at the anterior and posterior termini of the follicle that induce a terminal cell fate on their neighboring FC (Beccari et al., 2002; Xi et al., 2003). The third subtype, the cuboidal FC, form an epithelium that surrounds the cyst. At mid-oogenesis, cuboidal FC are patterned by the combined activities of Epidermal Growth Factor Receptor (EGFR) and Decapentaplegic (Dpp) pathways that determine the size, shape and position of specific chorion structures such as the operculum and dorsal appendages (Dobens and Raftery, 2000; Peri and Roth, 2000).

In this paper, we report that Slmb plays several different roles in oogenesis: it is required in FC for normal morphogenesis of the germarium, for cyst encapsulation, for timely differentiation of FC into different subpopulations and for chorion patterning. We present evidences that Slmb downregulates the Dpp pathway in FC, suggesting that most of the above phenotypes might be caused by overactivation of this pathway. In addition, we show that *slmb* loss of function in the germline provokes misregulation of cystocyte divisions, nurse cell endoreplication defects and incomplete dumping. These germline phenotypes correlate with a sharp decrease in the levels of the E2f subunits E2f2 and Dp, suggesting that Slmb participates in the regulation of the network of cell cycle modulators.

Materials and methods

Fly strains

The following *slmb* alleles were used: $slmb^{00295}$ (provided by T. Xu), $slmb^{1}$ and $slmb^{2}$ (provided by J. Jiang). The following markers were incorporated or recombined into $slmb^{2}$ *P{neoFRT82B}/TM3B Sb* genotype: $slbo^{01310}$ (Montell et al., 1992), PZ80 and dad^{1883} (obtained from A. Spradling). For overexpression studies, the following lines were used: *K25sevHs-gal4* (strong); *Hs-gal4* (weak); *CY2-gal4* (Queenan et al., 1997); *T155-gal4*, *e22C-gal4* (Duffy et al., 1998); *UAS-dpp* (strong) (Ruberte et al., 1995); *UAS-dpp* (weak) (Staehling-Hampton and Hoffmann, 1994); *UAS-medea* (Marquez et al., 2001); *UAS-activated tkv* (provided by S. Cohen) and *UAS-slmb* (Grima et al., 2002). sog^{U2}, dad^{j1E4}, $CSN5^{L4032}$, $dCul1^{12764}$ and $dCul1^{10494}$ lines were obtained from the Bloomington *Drosophila* Stock Center. Canton-S was used as wild type.

Induction of constructs and mosaic clones

For Gal4/UAS induction, flies were grown at 18°C until eclosion and then transferred to 25°C. Heat shocks were performed for 20 minutes at 37°C on three consecutive days. Eggs from these flies were collected or females were dissected 2-6 days after the last heat shock. Mutant clones were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993). Briefly, females *y w;* $slmb^{00295,\ 1\ or\ 2}$ *P{neoFRT82B}/TM3B Sb;* were mated with males *y w P{Hs-FLP}; P{neoFRT82B} P{w[+mC]=ovo*^{D1}*}3R/TM3* to generate germline clones (Chou and Perrimon, 1996); with males *w; P{en2.4-GAL4}e22c P{UAS-FLP1.D}JD1/CyO*; *P{neoFRT}82B* ry^{506} to generate follicle cell clones; or with males *w P{GawB}elav[C155], P{UAS-eGFP} P{Hs-FLP}; P{neoFRT}82B P{tubP-GAL80}LL3/TM6B* to generate positively marked GFP somatic clones (Lee et al., 2000).

Antibodies and cDNAs

For immunostaining, the following primary antibodies were used: rabbit anti-β-galactosidase (1/1200); anti-GFPmAB 3E6 (1/500) and rabbit anti-GFP (1/200; Molecular Probes); 7G10 anti-Fasciclin 3 (1/100, Developmental Studies Hybridoma Bank); and rabbit anti-Medea (1/500; a gift from Laurel Raftery). For western blot, the following antibodies were used: guinea pig anti-E2f1 (1/2000; a gift from Terry Orr-Weaver); anti-E2f2 mei8 (1/2) and anti-Dp yun-6 (1/3) (Frolov et al., 2001); mouse monoclonal anti-CycE (1/10; a gift from Helena Richardson); and mouse anti-hsp70 (1/5000, Sigma). The secondary antibodies Cy3-conjugated donkey anti-mouse, Cy2-conjugated donkey anti-rabbit, donkey anti-mouse-HRP and goat anti-rabbit-HRP were from Jackson ImmunoResearch; anti-guinea pig-HRP was from Sigma. The *Broad-Complex* core domain cDNA (Deng and Bownes, 1997) was kindly provided by Mary Bownes.

Results

Slmb expression during oogenesis

As a first step in the analysis of Slmb function in oogenesis, we wanted to assess the cell types and stages in which the gene is expressed. We failed to detect the transcript by in situ hybridization, suggesting that *slmb* is expressed at very low levels. Although enhancer traps do not always reproduce all aspects of endogenous transcription, they might provide a useful and often more sensitive method for studying gene expression when transcripts cannot be detected directly. We used the $slmb^{00295}$ enhancer trap line (Jiang and Struhl, 1998) that revealed a widespread and dynamic expression profile of *slmb* during oogenesis. High levels of expression were observed in nurse cells all throughout oogenesis (Fig. 1A). From the germarium until stage 8 of oogenesis, expression was excluded from FC (Fig. 1B-D) and at stage 9, low β-galactosidase levels could be detected (Fig. 1E). From stage 10 onwards, strong expression was recorded in patches of FC surrounding the oocyte (Fig. 1F), being still detected by the end of oogenesis (not shown). Remarkably, at stage 11 very strong expression was observed in two dorsal patches of FC that give rise to dorsal appendages (Fig. 1G) (Deng and Bownes, 1997; Sapir et al., 1998; Wasserman and Freeman, 1998). As the expression pattern suggested a function for *slmb* in both the germline and FC, we wanted to determine whether *slmb* loss of function provokes defects in oogenesis.

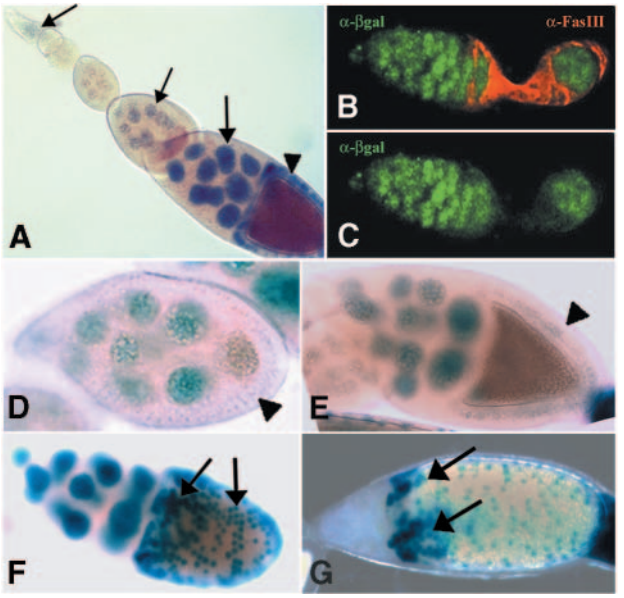

Fig. 1. *slmb* shows a dynamic expression pattern during egg chamber development. Ovaries from females carrying the $slmb^{00295}$ enhancer trap were analyzed by X-gal staining or by anti-β-galactosidase (anti-β-gal) immunofluorescence. (A) In the ovariole, nurse cells stain positive for X-gal throughout oogenesis (arrows). X-gal signal in FC surrounding the oocyte appears at mid-oogenesis (arrowhead). (B,C) In the germarium, expression of β-gal (green) is restricted to the germline and is excluded from FC that express Fasciclin 3 (red). (D) Expression of β-gal cannot be detected in FC at stage 8 (arrowhead) and starts at stage 9 (E, arrowhead). (F) At oogenesis stage 10, strong expression of the enhancer trap occurs in a scattered pattern in the follicular epithelium (arrows). (G) At stage 11, expression becomes stronger in two patches of FC that will form the dorsal appendages (arrows).

Slimb is required in both germline and follicle cells for egg chamber development

We tested three different *slmb* alleles: $slmb^{1}$, previously described as a hypomorphic allele; and $slmb^{2}$ and $slmb^{00295}$, reported to be null alleles (Jiang and Struhl, 1998; Theodosiou et al., 1998). As in all cases homozygous or heteroallelic combinations were lethal at different developmental stages (Jiang and Struhl, 1998), we decided to examine the effect of *slmb* loss of function in homozygous germline clones generated by the FLP/Ovo^{D} method (Chou et al., 1993; Chou and Perrimon, 1996) or in somatic clones induced with the FC driver e22C (Duffy et al., 1998) (see Materials and methods). $slmb^{00295}$ clones were recovered at low rates, probably owing to poor cell viability (Jiang and Struhl, 1998); $slmb^{1}$ and $slmb^{2}$ alleles exhibited similar highly reproducible egg chamber defects and were used in most of the experiments throughout this study. DAPI staining of egg chambers bearing germline clones revealed that 17.4% of the follicles (n=483) exhibited different kinds of abnormalities. To rule out the possibility that some of the observed defects arise from an effect of the Ovo^{D} itself, we focused our phenotypic analysis on ovarioles with vitellogenic cysts, a situation never found in ovaries from females carrying one copy of the Ovo^{D} transgene (n=100 ovarioles). Germline phenotypes included follicles that degenerated, exhibiting features of apoptosis (3.7%, Fig. 2A,B), such as germ cell nuclear fragmentation (Fig. 2C); consistent with this, TUNEL-positive staining at early stages of oogenesis was recorded (data not shown). Another frequently observed phenotype consisted of follicles displaying either increased (9.9%) or reduced (1.7%) numbers of germ cells (Fig. 2D-F), as well as germ cell nuclei that were heterogeneous in size (2.9%, Fig. 2G). These results suggest that mitotic divisions, as well as the switch from mitosis to endoreplication, might be misregulated in these cysts (see below). Remarkably, in egg chambers with a reduced number of germ cells, the nuclei were bigger than normal (see below Fig. 3E), suggesting that these cells failed to divide and, instead, entered endoreplication prematurely. In another set of experiments, we analyzed the effect of *slmb* loss of function in FC clones. Although $slmb^{00295}$ enhancer trap analysis indicated that expression in FC apparently starts at stage 9 (Fig. 1E), we frequently observed two oocytes and extra nurse cells within a single follicular epithelium (Fig. 2H). At vitellogenic stages, we never found supernumerary follicles exhibiting one single oocyte, suggesting that the phenotype arise from encapsulation defects rather than from an extra round of mitosis in the cyst. Consistent with encapsulation defects, we observed that morphology of mutant germaria was altered: in wild-type germaria region IIb, the cyst has acquired a lens shape, spanning the whole width of the germarium (Fig. 2I); instead, *slmb* mutant ovarioles often exhibited germaria in which two or more cysts were placed side by side in regions IIb and III (Fig. 2J). In addition, egg chambers with somatic clones often displayed an aberrant shape (Fig. 2K), suggesting that normal formation of the follicular epithelium was impaired. Moreover, we observed that oocytes were frequently mispositioned within the follicle, adopting an anterior (Fig. 2L) or lateral (Fig. 2M) location, indicating that axial polarity of the egg chamber was altered. Finally, another category of phenotypes consisted of ovarioles lacking interfollicular stalks between adjacent follicles but separated, instead, by a layer of epithelial cells (Fig. 2N,O). Previous work has shown that posterior localization of the oocyte depends on the presence of a stalk that links the egg chamber with the neighboring older follicle (Torres et al., 2003). We observed that in some of the follicles with mislocalized oocytes, posterior interfollicular stalks were missing (9/15), while in others posterior stalks were present (6/15), suggesting that alterations in axial polarity in the latter egg chambers involved a different mechanism.

As Slmb is a substrate recognition subunit of an SCF complex (Bocca et al., 2001; Feldman et al., 1997; Skowyra et al., 1997; Yaron et al., 1998), mutations affecting other components of the complex or molecules required for its ubiquitin ligase activity should render overlapping biochemical effects and similar phenotypes in oogenesis. We induced *cullin1* (*lin19* – FlyBase) ($dCul1^{10494}$) (Bocca et al., 2001; Filippov et al., 2000; Heriche et al., 2003; Khush et al., 2002) general clones by the Hs-FLP/FRT method that rendered ovarioles with cyst encapsulation defects, lack of interfollicular stalks and nurse cell nuclei of heterogeneous size (data not shown). Moreover, mutations affecting the subunits CSN4 and CSN5 of the COP9 signalosome, a highly conserved complex that regulates the activity of SCF complexes (Bech-Otschir et al., 2002; Schwechheimer and Deng, 2001; Seeger et al., 2001), have recently been reported to cause phenotypes that are also very similar to those provoked by *slmb* mutations

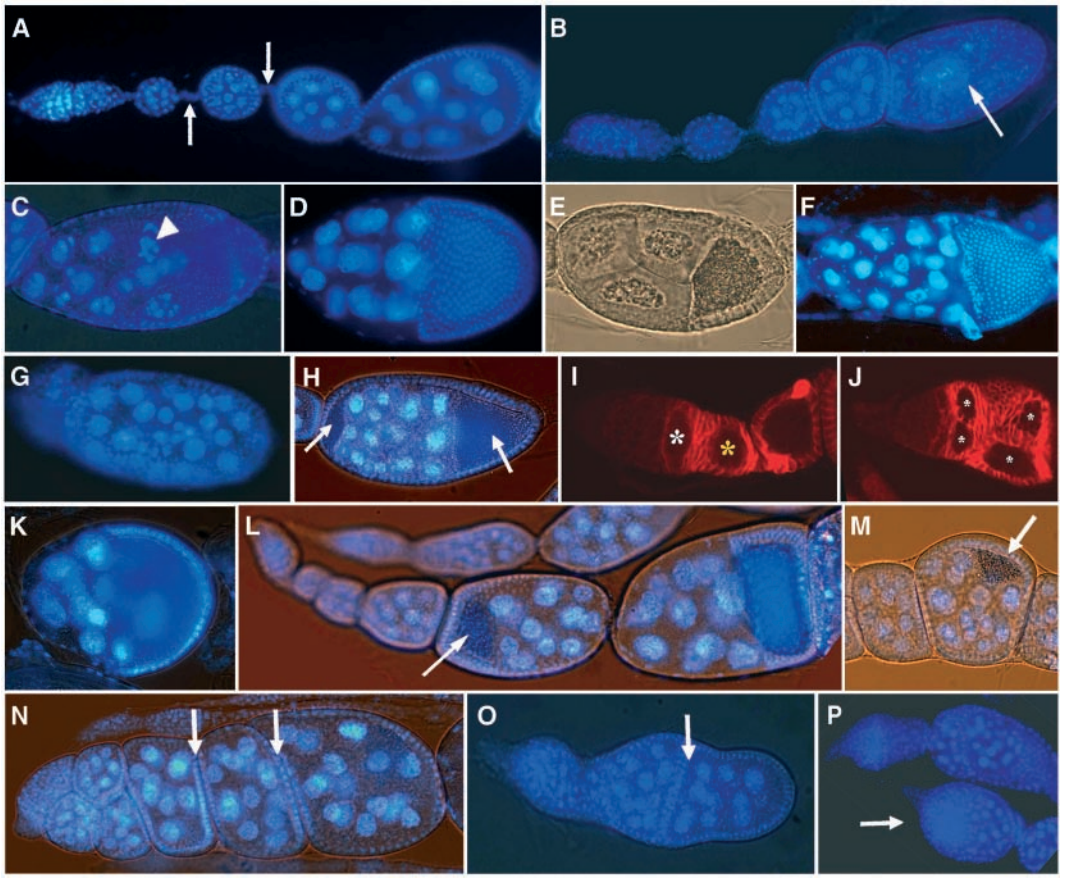

Fig. 2. *slmb* loss of function provokes a wide array of ovarian phenotypes. (A) DAPI staining of a wild-type ovariole (interfollicular stalks are marked by arrows). (B) Some egg chambers from females with *slmb* germline clones exhibit premature apoptosis (arrow) indicated by the occurrence of fragmented nurse cell nuclei (C, arrowhead). (D) Wild-type follicles always contain 15 nurse cells and one oocyte; in *slmb* germline clones, egg chambers with decreased (E) or increased (F) number of germ cells are frequently observed, whereas in other follicles germ cell nuclei were heterogeneous in size (G). (H) In females bearing FC clones, egg chambers with two oocytes occur (arrows). (I) Wild-type germarium in which FC are stained for Fas3; the cyst in region IIb shows the typical lens shape (white asterisk) and once in region III the cyst re-shapes into a sphere (yellow asterisk). (J) In *slmb* somatic clones, the general shape of the germarium is frequently altered and cysts are placed side by side in regions II and III (asterisks). *slmb* FC clones exhibit a wide array of defects, including abnormal morphology of egg chambers (K; compare with D), oocytes that are mispositioned (L,M, arrows) and lack of interfollicular stalks between adjacent follicles (N,O, arrows; compare with A). (P) *slmb/CSN5* genetic interactions; $slmb^2$ germline clones that are heterozygous for $CSN5^{L4032}$ display enhanced phenotypes such as enlarged germaria (arrow).

(Doronkin et al., 2002; Doronkin et al., 2003). To study if Slmb might be a component of an SCF complex in the ovary, we looked for genetic interactions in egg chambers with $slmb^2$ germline clones that were at the same time heterozygous for $dCul1^{12764}$ or $CSN5^{L4032}$ alleles. Strong interactions were detected, as the proportion of egg chambers with extra germ cells increased from 9.9% (n=483) in ovaries with $slmb^2$ homozygous clones to 32.1% in those with $dCul1^{12764}$/+; $slmb^2/slmb^2$ clones (n=156) and to 25.4% in ovaries with $CSN5^{L4032}$/+; $slmb^2/slmb^2$ clones (n=130). Moreover, the phenotypes were enhanced in these genotypic combinations, as egg chambers with more than 32 germ cells and enlarged germaria (Fig. 2P) were observed in addition to the phenotypes described above. These results suggest that Slmb functions as a component of an SCF complex in oogenesis.

Control of germ cell divisions is impaired in *slimb* germline clones

Phenotypes described in Fig. 2E,F suggested that *slmb* loss of function in the germline might cause an impairment in the regulation of mitotic divisions that originate the cyst (de Cuevas et al., 1997). Among follicles with extra germ cells (n=38), two types of egg chambers could be recognized: those with one single oocyte (16/38) and those with two oocytes (22/38). In order to determine if extra gem cells arise from an additional round of mitotic divisions, we analyzed the pattern of ring canals in these cysts. In wild-type cysts, four rounds of mitotic divisions take place, giving rise to oocytes with four ring canals clearly visible by phalloidin staining (Fig. 3A) (Hawkins et al., 1996). By contrast, in *slmb* germline clones, all oocytes from follicles with extra nurse cells and one single oocyte exhibited five ring canals instead of four (16/16; Fig. 3B), indicating that an extra round of mitosis occurred. However, all the observed egg chambers bearing two oocytes exhibited four ring canals per oocyte (22/22; Fig. 3C,D), suggesting that in these cases mitotic divisions were normal. We believe that the latter phenotype did not reflect *slmb* loss of function in the germline but instead was due to the occurrence of *slmb* FC clones that provoked defects in cyst encapsulation as described above. Interestingly, egg chambers with fewer than 16 germ cells (n=8) exhibited a number of ring canals that was always consistent with the predicted rounds of cell divisions (i.e. two cells=one ring canal; four cells=no more than two ring canals per cell, etc.) (Fig. 3E). From these experiments, we hypothesized that Slmb is required in the germline for the regulation of mitotic cycles during cyst formation.

It has been reported that *Drosophila* Dp (Dp), which forms heterodimers with E2f transcription factors (E2f1 and E2f2), is required for essential processes during oogenesis. *Dp* mutant

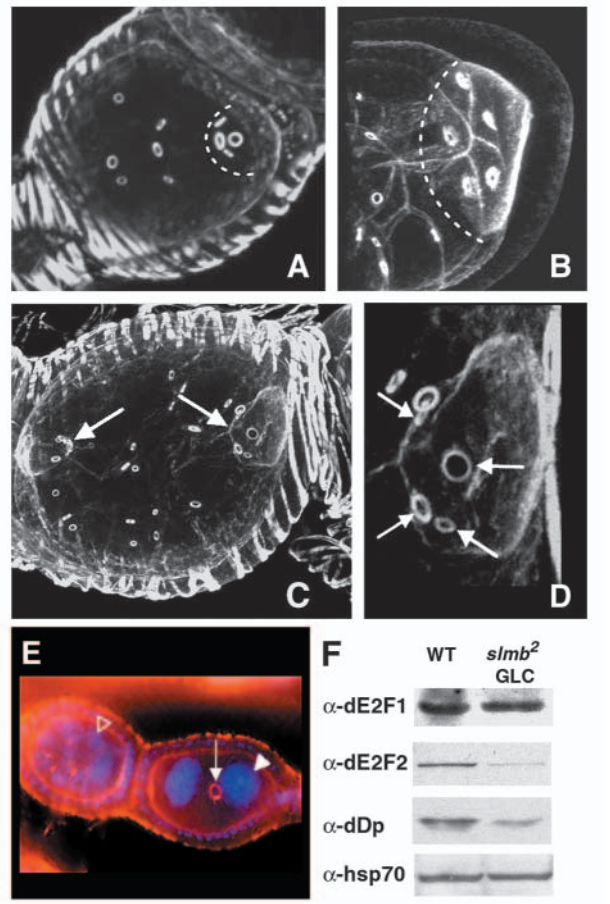

Fig. 3. Germ cell mitosis is misregulated in *slmb* germline clones. Ring canals are visualized by TRITC-Phalloidin staining. (A) In wild-type egg chambers, the oocyte (marked with a broken line) connects to neighboring nurse cells through four ring canals. (B) An oocyte (broken line) from a *slmb* germline clone exhibits five ring canals, indicating that an extra round of mitosis has occurred. (C) *slmb* mutant egg chamber with extra germ cells and two oocytes (arrows). (D) Higher magnification image of the oocyte on the right in C, showing that in these follicles, oocytes exhibit four ring canals (arrows). (E) In a *slmb* mutant ovariole, the egg chamber on the right has only two germ cells connected by a single ring canal (arrow) and exhibit very big nuclei (white arrowhead); in the same ovariole, the egg chamber on the left has the normal complement of germ cells and their nuclei are much smaller (open arrowhead). (F) Western blot analysis of E2f complex components in ovarian extracts with *slmb* germline clones (GLC), compared with those of wild-type ovaries; whereas E2f1 does not show obvious differences, Dp and E2f2 were clearly reduced in *slmb* mutant ovaries.

germline clones often execute an extra round of mitosis, giving rise to follicles with extra nurse cells (Myster et al., 2000; Royzman et al., 2002; Taylor-Harding et al., 2004). To gain insights about the mechanism underlying misregulation of the cell cycle in *slmb* germline clones, we performed western blot analysis of Dp, E2f1 and E2f2. As can be seen in Fig. 3F, E2f1 protein levels were not affected in *slmb* mutant ovaries but, by contrast, a major decrease in E2f2 and Dp levels occurred. As a Slmb direct target is expected to be increased in *slmb* mutant clones, we believe that the reduction of Dp and E2f2 probably occurs through an indirect mechanism.

Slimb somatic clones cause defects in differentiation of follicle cells and generate ectopic polar cells

In the germarium, a lineage of intercyst FC differentiates into two subpopulations that stop dividing and originate stalk and polar cells (Bai and Montell, 2002; Margolis and Spradling, 1995; Tworoger et al., 1999). The lack of interfollicular stalk cells observed in *slmb* loss-of-function ovarioles suggested that differentiation of FC into different subpopulations might be impaired. In wild-type germaria, undifferentiated FC express high levels of Fasciclin 3 (Fas3), and by oogenesis stage 4, expression becomes restricted to polar cells (Fig. 4A). In *slmb* somatic clones refinement of Fas3 expression was often delayed (Fig. 4B), being still detected in large patches of cells beyond oogenesis stage 9 (Fig. 4C). In order to examine cell autonomy of this effect, clones positively marked with GFP were generated by using the MARCM system (Lee et al., 2000). As can be seen in Fig. 4C-E, Fas3 labeling largely overlaps with cell patches expressing GFP, indicating that the effect is cell autonomous. In some mosaic egg chambers, Fas3 expression refined into a pattern that seemed to correspond to ectopic polar cells and, interestingly, these cells were mutant for *slmb*, as indicated by GFP positive staining (Fig. 4C-E, arrowheads). As Fas3 is not a specific polar cell marker (Lopez-Schier and St Johnston, 2001; Zhang and Kalderon, 2000), we used the PZ80 enhancer trap that is specifically expressed in these cells (Fig. 4F-H) (Karpen and Spradling, 1992; Spradling, 1993). In wild-type follicles, anterior polar cells induce differentiation of five to eight FC into a border cell fate. The whole border cell cluster, including polar cells, expresses the *slow border cells* gene (*slbo*), as revealed by expression of the *slbo-lacZ* enhancer trap (Fig. 4I) (Grammont, 2002; Liu and Montell, 1999; Montell et al., 1992; Rorth et al., 2000). At oogenesis stage 9, polar cells surrounded by the whole cluster start migrating posteriorly between nurse cells (Fig. 4G), and by stage 10 they have reached the anterior border of the oocyte (Fig. 4H). In *slmb* mutant egg chambers we clearly observed the occurrence of ectopic polar cells (Fig. 4J) and consistent with this, ectopic clusters of border cells differentiated (Fig. 4K). Interestingly, migration of border cell clusters was often impaired in these follicles (Fig. 4J,K).

Slimb is required for chorion patterning

Eggs laid by females in which *slmb* mutant clones were induced with the *Hs-FLP* driver exhibited diverse chorion patterning defects. Some of them, like a smaller size or abnormal shape of the egg (Fig. 5A; data not shown) probably derive from dumping defects or from alterations in the general morphology of the egg chamber (Fig. 2K). Interestingly, we also observed a wide array of eggshell phenotypes in which the size, shape and/or position of dorsal appendages (DA) was altered. In wild-type eggs, the dorsoanterior region of the chorion displays a pair of filaments (DA) separated by a gap (Fig. 5B). Some eggshells derived from *slmb* mosaic ovaries had a dorsalized phenotype (Sapir et al., 1998; Wasserman and Freeman, 1998) characterized by laterally expanded DA (Fig. 5C) or in more severe cases, by DA surrounding all the anterior circumference of the egg (Fig. 5D). In other cases, ectopic DA formed (Fig. 5E) or DA deranged, giving rise to ramified structures (Fig. 5F). Another category of phenotypes was that of eggshells apparently ventralized (Schupbach, 1987;

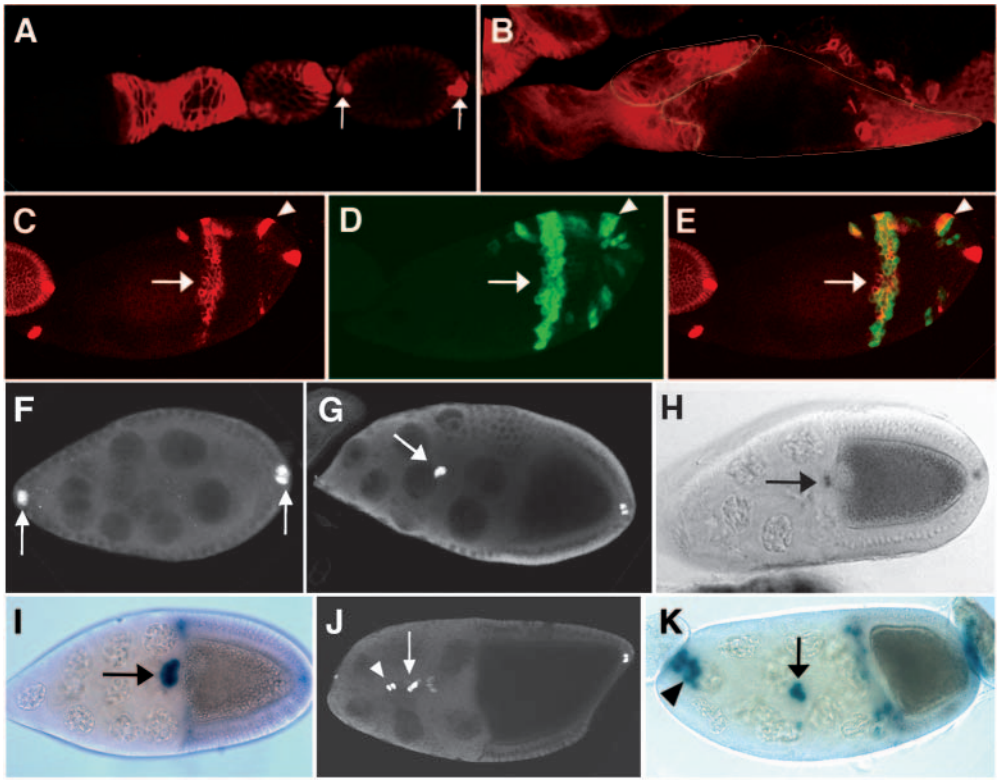

Fig. 4. Delayed FC differentiation and ectopic polar cells occur in *slmb* somatic clones. (A) In wild-type ovarioles, Fas3 expression (red) refines as oogenesis progresses and from stage 4 onwards becomes restricted to polar cells (arrows). (B) In *slmb* FC clones, Fas3 remains widespread (egg chambers are outlined). (C) In egg chambers bearing *slmb* FC clones, large patches of cells expressing Fas3 are still detected at stage 9 (arrow). Mutant clones from the egg chamber shown in C were positively marked with GFP (D, arrow) and the merged confocal image shows that ectopic Fas3 correlates with the position of the clones (E, arrow). Arrowheads in C-E mark a pair of cells that are probably ectopic polar cells. (F-H,J) The PZ80 enhancer trap is specifically expressed in polar cells. In wild-type ovaries at stage 8, the two pairs of polar cells are localized at the anterior and posterior termini of the follicle (F, arrows) at stage 8; at stage 9, anterior polar cells migrate posteriorly between nurse cells (G, arrow); and at stage 10, they have reached the anterior border of the oocyte (H, arrow). (I,K) The *slbo-LacZ* element is expressed in border cells. In stage 10 wild-type follicles, border cells can be seen at the anterior end of the oocyte (I, arrow). Egg chambers bearing *slmb* FC clones often exhibit extra polar cells (J, arrowhead) and differentiate ectopic clusters of border cells (K, arrowhead). In these follicles, border cell migration is often delayed and by stage 10, they are located between nurse cells (J,K, arrows).

Wasserman and Freeman, 1998), with DA partially or totally fused (Fig. 5G,H). Finally, in the last category, eggshells exhibited a slightly enlarged operculum [with DA shifted to a more posterior site (Fig. 5I)] or a greatly reduced amount or absence of DA material (Fig. 5J,K).

In order to distinguish which chorion phenotypes arise from *slmb* loss of function in the germline and which ones from clones in the follicular epithelium, we induced clones through the Ovo^D method or the FC driver e22C. The latter method caused the same DA phenotypes described above, indicating that abnormalities reflected *slmb* loss of function in the follicular epithelium. By contrast, eggs of smaller size, which derived from dumping defects (data not shown), were not observed upon induction of somatic cell clones but rather, were laid by females with germline clones (8%; n=902), indicating that this phenotype was due to Slmb loss of function in the germline.

Mild overexpression of Dpp in follicle cells mimics *slimb* loss-of-function phenotypes

It has been reported that high levels of Dpp at the anterior-most region of the chorion prevent DA formation, leading to differentiation of the operculum (Peri and Roth, 2000; Twombly et al., 1996). As eggshells derived from follicles with *slmb* somatic clones exhibited phenotypes resembling those that occur upon overactivation of the Dpp pathway (Fig. 5I), we decided to perform further ectopic Dpp expression experiments and look for novel eggshell phenotypes and for abnormalities

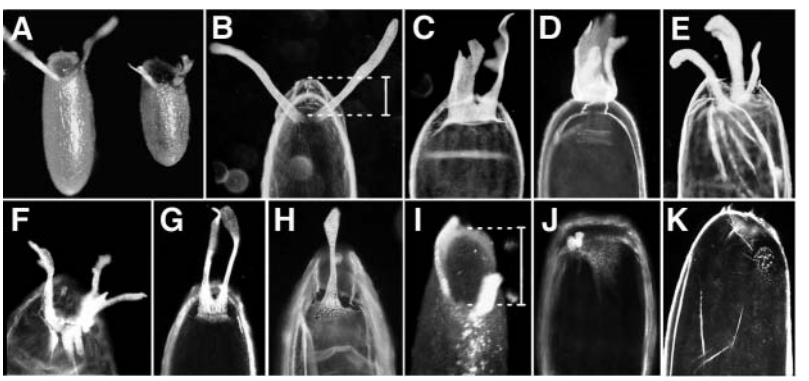

Fig. 5. *slmb* mutant clones cause chorion patterning defects. Eggshells were visualized by reflected light or dark-field microscopy. (A) Eggs laid by females with *slmb* germline clones (right) are often much smaller than wild-type eggs (left). (B) Wild-type dorsal appendage (DA) pattern. (C-F) Egg chambers with *slmb* somatic clones generate eggshells with variable DA abnormalities, including dorsalized phenotypes (C-E), ramified DA (F), ventralized phenotypes (G,H), and DA shifted to a more posterior position (I), reduced (I,J) or absent (K). In cases where DA are shifted posteriorly (I), the operculum appears enlarged when compared with wild-type eggshells (B); the limits of the opercula are indicated with broken lines.

Table 1. Chorion phenotypes obtained upon overactivation of the Dpp pathway

Maternal genotype	Chorion phenotypes ($n \geq 120$)
Hs-gal4 (strong)/+; *UAS-dpp* (weak)/+	No viable adults were obtained
Hs-gal4 (weak)/+; *UAS-dpp* (strong)/+	89% of eggshells with giant opercula and reduced or absent dorsal appendages (DA).
CY2-gal4/+; UAS-dpp (weak)/+	80% of eggshells with enlarged opercula and reduced DA.
T155-gal4/+; UAS-dpp (weak)/+	76% of eggshells with enlarged opercula and reduced or malformed DA. Weaker phenotypes than with the CY2 driver.
Hs-gal4 (weak)/*UAS-dpp* (weak)	38% of eggshells with variable phenotypes, including ventralized or fused DA and ectopic DA material.
e22C-gal4/UAS-dpp (weak)	17% of eggshells displaying abnormal DA morphology, such as ramified, fused or expanded DA.
Hs-gal4 (weak)/*UAS-dpp* (weak) (no heat shock, leaky overexpression)	29% of eggshells with ectopic DA material deposited at the base, often displaying shorter or malformed DA.
sog^{U2}/+	2% of eggshells with shorter DA and ectopic material deposited at the base and 9% with fused or partially fused DA.
dad^{j1E4}/+	3% of eggshells with shorter DA and ectopic material deposited at the base.
CY2-gal4/UAS-slmb; +/+	All wild-type egg chambers.
CY2-gal4/UAS-slmb; UAS-dpp (weak)/+	All wild-type egg chambers.

in egg chamber development. We used various Gal4 drivers and UAS constructs for inducing the Dpp pathway at different levels. At 25°C all crosses were lethal at pre-imaginal stages and only at 18°C some of the tested combinations rendered viable adults that allowed ovary and chorion analysis. As depicted in Table 1, in the strongest viable combinations overexpressing Dpp, opercula expanded dramatically and DA were absent (Fig. 6A) as was previously reported (Peri and Roth, 2000; Twombly et al., 1996). In these cases, we observed germaria with tumorous arrays of germline stem cells (not shown) (Xie and Spradling, 1998) as well as aberrant follicles with large numbers of germ cells engulfed in the same follicular epithelium (Fig. 6B). Interestingly, upon milder induction of the Dpp pathway, we observed variable DA phenotypes, very similar to those caused by *slmb* loss of function in somatic cells (Table 1). These included eggshells exhibiting one thin dorsal filament (Fig. 6C), fused or ramified DA (Fig. 6D,E), DA spanning the whole anterior circumference of the egg (Fig. 6F) and reduced DA (Fig. 6G). Similar phenotypes were observed (1) upon overexpression of an activated form of the type I receptor Thick veins (not shown); (2) by overexpressing the co-Smad protein Medea (Fig. 6H); and, (3) in low proportion, in eggs laid by females heterozygous for *short gastrulation* (*sog*) or for *daughters against dpp* (*dad*) genes (Fig. 6I; Table 1), which encode negative regulators of the pathway (Araujo and Bier, 2000; Francois et al., 1994; Tsuneizumi et al., 1997). When we analyzed ovarioles from females subjected to the same mild overexpression conditions of Dpp, we observed a wide array of phenotypes similar to those observed in *slmb* mutant somatic clones. These included abnormal germaria, two complete cysts encapsulated within a single follicular epithelium, mislocalized oocytes and lack of interfollicular stalks between adjacent follicles (Fig. 6J-M). In order to determine whether loss of Slmb might cause increased activation of the Dpp pathway leading to the above phenotypes, we initially tested whether overexpression of Slmb was able to antagonize the effect of ectopic Dpp on eggshell morphogenesis. Indeed, Slmb overexpression rendered full

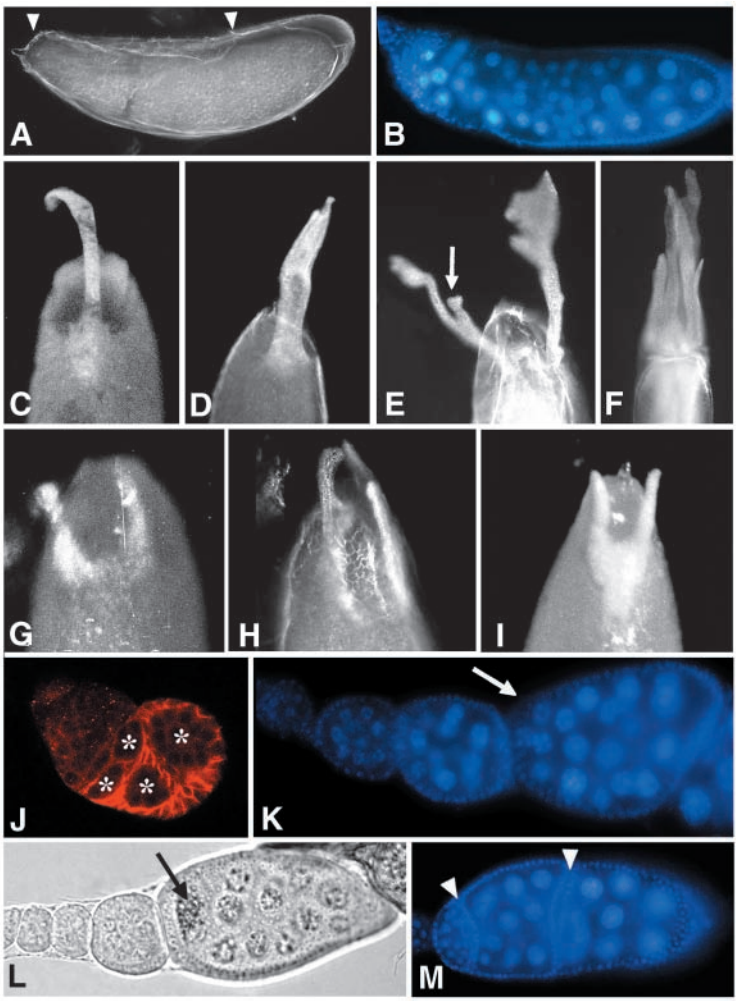

Fig. 6. Mild overactivation of the Dpp pathway mimics *slmb* mutant phenotypes. (A) Eggs laid by females bearing the weak *Hs-gal4* driver and the strong *UAS-dpp* element that were subjected to heat shock show a dramatic expansion of the operculum (marked by arrowheads) and lack of DA. (B) DAPI staining of an ovariole dissected from one of these females reveals that many germ cells are surrounded by a single follicular epithelium. (C,D) Upon milder overexpression using the weak *UAS-dpp* element and the weak *Hs-gal4* driver, ventralized eggshells with a single DA occurred. (E,F) Variable chorion phenotypes resulting from overexpression with the same *UAS-dpp* element driven by *e22C-gal4*: DA can be ramified (E, arrow), expanded (F) or greatly reduced (G). DA reduction was also observed upon overexpression of Medea with the weak *Hs-gal4* driver (H) and in eggs laid by heterozygous sog^{U2} females (I). Mild overexpression of Dpp with the weak *Hs-gal4* or *e22C-gal4* drivers (J-M) produced germaria with abnormal morphology (J), egg chambers with extra germ cells (K, arrow), mispositioned oocytes (L, arrow) and ovarioles lacking interfollicular stalks (M, arrowheads).

reversion of the 'expanded operculum' phenotype (*n*=376; Fig. 6A) of eggs laid by females overexpressing Dpp under control of a *CY2-gal4* driver (Table 1); no rescue was observed when an *UAS-EGFP* transgene was overexpressed instead. Thus, based on similarities of *slmb* loss-of-function phenotypes with those caused by Dpp mild overexpression and, considering that the strong ectopic Dpp eggshell phenotype could be rescued by simultaneous overexpression of Slmb, we hypothesized that Slmb negatively regulates Dpp pathway in ovarian somatic cells.

Ectopic activation of the Dpp pathway occurs in *slmb* loss-of-function follicles

In stage 10 wild-type egg chambers, the intersection of Dpp and EGFR pathways induces the expression of the *Broad-Complex* (*BR-C*) gene in two patches of dorsoanterior FC (Fig. 7A), promoting DA specification and positioning (Deng and Bownes, 1997; Peri and Roth, 2000). We found that in egg chambers bearing *slmb* FC clones the *BR-C* pattern was altered, being in some cases expanded (Fig. 7B), whereas in others it was reduced (Fig. 7C) or partitioned (Fig. 7D). As we did not detect alterations in the EGFR pathway (data not shown), we analyzed a possible effect of *slmb* mutations on Dpp signaling, by using the dad^{1883} enhancer trap (*dad-LacZ*) (Casanueva and Ferguson, 2004). In wild-type ovarioles, expression of *dad-lacZ* is first detected at stage 8 in terminal anterior FC that migrate towards the oocyte (Fig. 7E). By oogenesis stage 10, *dad-LacZ* expression is detected in centripetal and border cells located at the anterior border of the oocyte, as well as in stretched cells which surround the nurse cells (Fig. 7F). Analysis of *dad-lacZ* in ovaries bearing *slmb* mutant clones revealed that expression started prematurely at stage 6 (Fig. 7G) and later on, at stages 9-10, the pattern was often expanded posteriorly (Fig. 7H,I). These results suggest that loss of *slmb* might cause ectopic activation of the Dpp pathway. The ubiquitin/proteasome system regulates the stability of various Smad proteins through the activity of different E3 ubiquitin ligases (Casanueva and Ferguson, 2004; Fukasawa et al., 2004; Fukuchi et al., 2001; Li et al., 2004; Liang et al., 2004; Wan et al., 2004). Because in mammalian cell culture proteasomal degradation of Smad4, a common signal transducer in the TGFβ signaling pathway, is regulated by the Slmb ortholog, βTrcp1 (Wan et al., 2004), we decided to explore whether a similar mechanism operates in the *Drosophila* ovary. We initially analyzed the expression of the *Drosophila* Smad4 homologue Medea (Sutherland et al., 2003) in wild-type follicles. At stage 8-9, expression was restricted to migrating anterior cells (Fig. 7J) and at stage 10 expression could be observed in stretched cells as well as in all FC surrounding the oocyte, being remarkably stronger in the centripetally migrating FC, located at the anterior border of the oocyte (Fig. 7K). Interestingly, in egg chambers bearing *slmb* clones, ectopic patches expressing high levels of Medea were observed (Fig. 7L,M). Taken together, these results suggest that at least some of the *slmb* mutant phenotypes might have been caused by increased levels of Medea protein, which led to overactivation of the Dpp pathway.

Discussion

Slimb function in the germline

We have shown that the F-box protein Slmb is required for

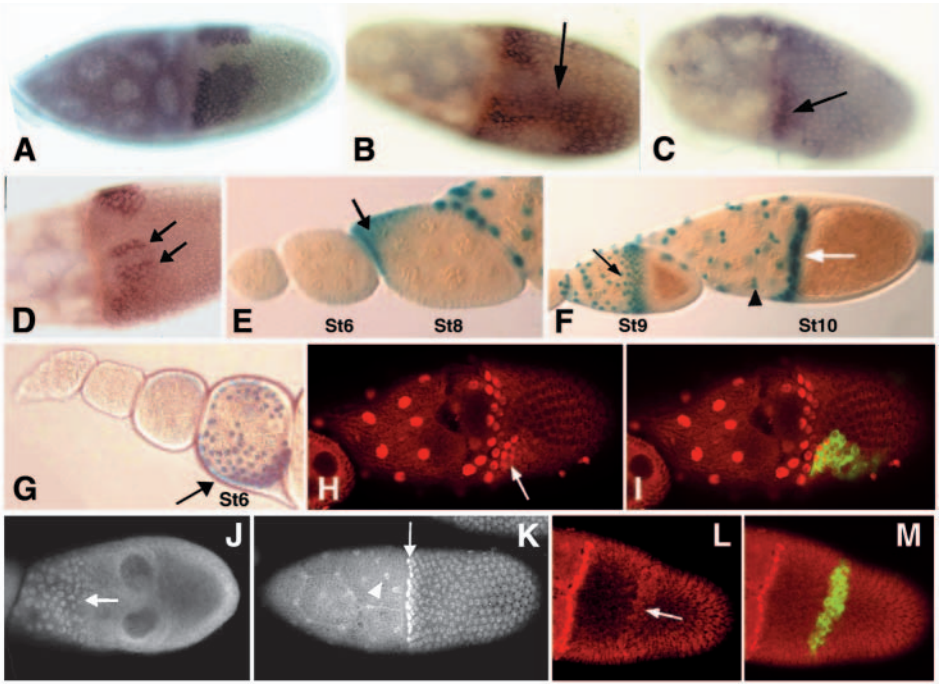

Fig. 7. *Broad-Complex* (*BR-C*) expression and ectopic activation of the Dpp pathway. (A) mRNA in situ hybridization in a stage 10 wild-type egg chamber showing that *BR-C* is expressed in two patches of dorsoanterior FC. (B-D) In follicles bearing *slmb* somatic clones, *BR-C* expression is in some cases expanded (B, arrow) and in others reduced (C, arrow) or split (D, arrows). (E,F) Expression of the *dad-lacZ* enhancer trap in wild-type ovarioles. (E) In the follicular epithelium, expression is first observed at stage 8 in anterior FC (arrow), being undetectable at stage 6. (F) At stage 9, *dad-lacZ* can be seen in migrating anterior FC (black arrow) and at stage 10 in stretched cells (arrowhead) and in centripetal cells localized at the anterior border of the oocyte (white arrow). (G) In ovarioles bearing *slmb* mutant clones, expression of *dad-lacZ* occurs prematurely at stage 6 (arrow); at stage 10 (H), expression of the enhancer trap often expands posteriorly (arrow). (I) Positive GFP labeling of a *slmb* mutant clone showing that GFP signal overlaps with ectopic expression of *dad-lacZ* in the clone. (J,K) In wild-type ovaries, expression of Medea occurs at stage 9 in anterior FC that migrate towards the oocyte (J, arrow) and at stage 10 (K) in all FC, being much stronger in stretched cells (arrowhead) and in centripetal cells localized at the anterior border of the oocyte (arrow). (L) In *slmb* mutant follicles, strong Medea signal was detected in ectopic patches of columnar FC, at stage 10 (arrow). (M) Positive GFP labeling of the *slmb* mutant clone overlaps with this patch.

oogenesis in both the germline and FC. In the germline, we found that Slmb plays a role in the control of mitotic cycles during cyst formation, in regulation of nurse cell endoreplication and in nurse cell dumping. Recent reports have demonstrated that Slmb can control cell cycle progression in different experimental settings. It has been reported that, following DNA replication, Slmb is required in larval wing discs for proteolysis of a specific cell cycle modulator: the transcription factor E2f1 (Heriche et al., 2003). Remarkably, the E2f complex was implicated in cell cycle control of ovarian germ cells, in nurse cell transition from polyteny to polyploidy and in dumping of the nurse cell content into the oocyte (Myster et al., 2000; Royzman et al., 2002). In this study, we found that two subunits of the E2f complex, Dp and E2f2, were downregulated in ovaries bearing *slmb* germline clones, while E2f1 did not change. Differences in Cyclin E levels, another cell cycle regulator involved in cyst formation (Doronkin et al., 2003; Lilly and Spradling, 1996), could not be detected in these clones (data not shown). We want to stress the good correlation that exists between the phenotypes observed in *slmb* germline clones (this study) and in *Dp* germline clones (Myster et al., 2000), as in both cases an additional round of cystocyte mitotic divisions occurs. In order to understand the molecular mechanism causing Dp and E2f2 reduction in *slmb* germline clones, a detailed analysis of the alterations of the network regulating the cell cycle is required.

Slimb function in follicle cells

Although expression levels in somatic cells in the germarium are too low to be detected through an enhancer trap or by in situ hybridization, loss-of-function experiments suggest that *slmb* is needed in these cells for normal morphogenesis of the egg chamber and for encapsulation of the cyst. In addition, our results suggest that Slmb is required for timely differentiation of FC that is reflected by the refinement of Fas3 expression; this is accompanied by the occurrence of ectopic polar cells, lack of interfollicular stalks and disruption of normal egg chamber polarity. Later in oogenesis, Slmb is expressed at high levels in FC surrounding the oocyte and participates in chorion patterning, contributing to define the shape and position of DA.

It has been reported that *slmb* mutant clones induce ectopic activation of the Hedgehog (Hh) pathway in limb discs (Jiang and Struhl, 1998; Theodosiou et al., 1998). Notably, some of the phenotypes observed upon *slmb* somatic clone induction were similar to those originated by overactivation of the Hh pathway in FC. These include a delay in FC differentiation, development of ectopic polar cells and mislocalization of the oocyte (Forbes et al., 1996a; Zhang and Kalderon, 2000). Nevertheless, excessive activation of the Hh pathway also causes FC over-proliferation that results in excess of undifferentiated somatic cells that form very long interfollicular stalks between egg chambers (Forbes et al., 1996a; Forbes et al., 1996b; Zhang and Kalderon, 2000). By contrast, we observed that *slmb* loss of function in FC caused a lack rather than an excess of interfollicular cells. Finally, dominant genetic interactions were not detected between *slmb* and negative regulators of Hh pathway and the *ptc-LacZ* enhancer trap, which was reported to be activated in FC by the Hh pathway (Forbes et al., 1996b), was not induced ectopically in *slmb* mutant clones (data not shown). These results indicate that, despite some similarities between *slmb* loss-of-function and *hh* gain-of-function phenotypes, Slmb is unlikely to be a negative regulator of Hh pathway during oogenesis.

In limb discs, Slmb was also reported to be a negative regulator of the Dpp pathway, although the molecular mechanism involved is still unclear (Miletich and Limbourg-Bouchon, 2000; Theodosiou et al., 1998). We have shown that mild overexpression of Dpp caused a wide spectrum of phenotypes that were largely coincident with those caused by *slmb* loss of function in FC. Supporting the idea that loss of *slmb* might cause hyperactivation of the Dpp pathway, the strongest chorion phenotypes originated by overexpression of Dpp were completely antagonized by simultaneous overexpression of Slmb in FC. Moreover, expansion of *dad-lacZ* expression occurred in *slmb* mutant follicles, further suggesting that ectopic induction of the Dpp pathway indeed occurs as a consequence of *slmb* loss of function. Consistent with this, we found that a downstream component of the Dpp pathway, the co-Smad protein Medea, was upregulated in *slmb* mutant egg chambers. Because in mammalian cell culture it was demonstrated that Smad4 is a direct target of the mammalian Slmb ortholog, βTrcp1 (Wan et al., 2004), we believe that Medea could be a direct target of Slmb. Further molecular work is required to assess whether this is indeed the case or if alternatively, the effect of Slmb on Medea is indirect.

We thank Maki Asano, Konrad Basler, Mary Bownes, Stephen Cohen, Nick Dyson, Jing Jiang, Bernardette Limbourg-Bouchon, Stuart Newfeld, Terry Orr-Weaver, Laurel Raftery, Helena Richardson, Francois Rouyer, Danny Segal, Benny Shilo, Allan Spradling, David Stein, Tian Xu, the Bloomington *Drosophila* Stock Center and the DSHB for stocks and/or antibodies. We thank Helena Araujo and Amir Sapir for technical advice; and Hernán López-Schier, Fernanda Ceriani, Lázaro Centanín and members of P.W.'s laboratory for discussion. Wellcome Trust Grant 070161/Z/03/Z, Universidad de Buenos Aires X411 and ANPCyT 01-10839. M.M. is a fellow and P.W. is a career investigator of CONICET.

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