Drosophila dMyc is required for ovary cell growth and endoreplication

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Summary

Although the *Myc* oncogene has long been known to play a role in many human cancers, the mechanisms that mediate its effects in both normal cells and cancer cells are not fully understood. We have initiated a genetic analysis of the *Drosophila* homolog of the Myc oncoprotein (dMyc), which is encoded by the *dm* locus. We carried out mosaic analysis to elucidate the functions of dMyc in the germline and somatic cells of the ovary during oogenesis, a process that involves cell proliferation, differentiation and growth. Germline and somatic follicle cells mutant for *dm* exhibit a profound decrease in their ability to grow and to carry out endoreplication, a modified cell cycle in which DNA replication occurs in the absence of cell division. In contrast to its dramatic effects on growth and endoreplication, dMyc is dispensable for the mitotic division cycles of both germline and somatic components of the ovary. Surprisingly, despite their impaired ability to endoreplicate, *dm* mutant follicle cells appeared to carry out chorion gene amplification normally. Furthermore, in germline cysts in which the *dm* mutant cells comprised only a subset of the 16-cell cluster, we observed strictly cell-autonomous growth defects. However, in cases in which the entire germline cyst or the whole follicular epithelium was mutant for *dm*, the growth of the entire follicle, including the wild-type cells, was delayed. This observation indicates the existence of a signaling mechanism that acts to coordinate the growth rates of the germline and somatic components of the follicle. In summary, dMyc plays an essential role in promoting the rapid growth that must occur in both the germline and the surrounding follicle cells for oogenesis to proceed.

Key words: Myc, *dmyc*, Proto-oncogene, Nurse cells, Follicle, Egg chamber, Gene amplification, Dacapo, Cyclin E, Polyploidization, Endoreduplication

Introduction

Since the identification of *v-myc* as the transforming gene of the avian myelocytomatosis virus oncogene 20 years ago (Colby et al., 1983), the Myc family of nuclear proteins has been the subject of extensive investigation. Studies of Myc, the prototypical member of this family of proteins, have focused primarily on its role as a potent oncogene. Increases in Myc function arising from gene amplification, chromosomal translocations and proviral insertions are thought to be associated with a large proportion of cancer deaths (Dang, 1999). Although it is generally accepted that Myc influences cell behavior by regulating transcription (see Grandori et al., 2000), it is still not known how Myc influences the development of cancer, nor is its normal role in the cell clearly understood.

Myc is an essential gene in vertebrates; mice mutant for *Myc* die during embryogenesis (Davis et al., 1993) and exhibit reduced cell numbers in many developing organs (Trumpp et al., 2001). The function of the Myc oncoprotein in vertebrate cells has been correlated with the promotion of cell cycle progression; for example, *Myc* expression is rapidly upregulated in tissue culture cells exposed to growth factors (Kelly et al., 1983), and overexpression of *Myc* can force some quiescent cells to enter into S phase (Eilers et al., 1991). In addition to influencing the proliferation of vertebrate cells, overexpression of *Myc* can inhibit terminal differentiation (Coppola and Cole, 1986), and in some cases drives cells into apoptosis (Prendergast, 1999).

The ability of Myc to participate in these diverse cellular processes is thought to depend on its function as a sequence-specific transcription factor of the basic region/helix-loop-helix/leucine zipper (bHLH/LZ) class (Kretzner et al., 1992). Myc acts as a transcriptional activator in heterodimers with the bHLH/LZ protein Max, with the two proteins interacting via their LZ domains. It is this Myc-Max dimer that binds to the E box DNA consensus site CACGTG through bHLH sequences (Amati and Land, 1994; Blackwood et al., 1992). Early studies of Myc transcriptional regulatory activity focused on identifying target genes that control cell cycle progression. However, an accumulating body of evidence now suggests that

Myc and its binding partners regulate the expression of a large number of genes that regulate diverse functions, including protein synthesis, apoptosis, and DNA and energy metabolism (Boon et al., 2001; Guo et al., 2000; Neiman et al., 2001; Orian et al., 2003; Schuldiner and Benvenisty, 2001; Watson et al., 2002). Because the transcriptional activation induced by Myc-Max heterodimers is relatively weak and variable, it has been difficult to confirm potential target genes. Furthermore, recent data suggests that the transcriptional activity mediated by Myc may arise from its ability to influence levels of histone modification and alter chromatin structure (Amati et al., 2001; Eisenman, 2001). Taken together, these studies illustrate the complexity of Myc function, and indicate that much remains to be learned about the normal role of Myc in the life of the cell.

To take advantage of the powerful genetics available in the *Drosophila* system to study the role of Myc proteins in development and growth, we and others have cloned the single gene encoding a Myc family protein in *Drosophila* (dMyc), and have shown that it corresponds to the *diminutive* (*dm*) locus (Johnston et al., 1999; Schreiber-Agus et al., 1997). Flies homozygous or hemizygous for dm^1 are small, with slender bristles, indicating that dMyc is required for organismal and cellular growth. In fly imaginal discs, a reduction in dMyc function results in defects in cell growth but not proliferation; these cells divide at apparently normal rates but fail to increase in mass (Johnston et al., 1999).

At the time dMyc was cloned, the hypomorphic dm^1 mutation was the only *dm* allele available. To elucidate the function of dMyc in *Drosophila*, we generated a strong *dm* allele, dm^2, that is homozygous lethal. Using this allele, which behaves genetically as a null mutation, we have characterized the role of dMyc in *Drosophila* oogenesis, a process that encompasses cell proliferation, growth and differentiation. Using mosaic analysis, we show that dMyc is specifically required for cell growth and DNA endoreplication in both the germline and the somatic follicle cells. In contrast to these defects, cell proliferation in both types of cell was unaffected by reduced dMyc activity. Furthermore, although their ability to endoreplicate was severely compromised, dm^2 mutant follicle cells appeared to carry out gene amplification normally. Finally, we show that when either the germline or the soma is mutant for dm^2, the entire follicle is severely delayed in its progression through oogenesis, which indicates that the growth of the germline and the somatic follicle cells is interdependent.

Materials and methods

Drosophila stocks

All stocks were maintained employing standard conditions and procedures. The stock carrying *Dp(1;2)51b*, used in the identification of lethal mutant alleles of *dm*, was obtained from the Umea *Drosophila* Stock Center. The X-chromosome-linked insertion of the *D. melanogaster* variant H2A.F/Z class histone fused to the green fluorescent protein (hGFP) (Clarkson and Saint, 1999), used in the identification of germline and follicle cell mitotic clones, was a gift of Dr Joe Duffy. Other stocks used in the investigations reported below were obtained from the *Drosophila* Stock Centers in Bloomington, Umea and Toulouse. A description of mutant alleles, genetic markers and chromosome balancers used in this work can be found at FlyBase (http://flybase.bio.indiana.edu).

Screen for lethal alleles of *dm*

To isolate lethal mutations in *dm*, we employed a strategy that would enable us to specifically select for X-linked lethal mutations that are rescued by the presence of *Dp(1;2)51b*, which carries the *dm* locus as well as several neighboring loci, including the *white (w)* gene. *y w* males were mutagenized with 25 mM ethyl methansulfonate and mated to *C(1)DX, y w f; Dp(1;2)51b/+* females. 2763 F1 males of the genotype *y w */ Y; Dp(1;2)51b/+*, (where * represents a newly mutagenized chromosome), were mated individually to *C(1)DX, y w f; Dp(1;2)51b/+* females. 1823 of these crosses produced progeny, and these were scored for the presence or absence of w^- males [lacking Dp(1;2)51b]. The presence of w^- males indicated that the X chromosome carried no lethal mutations, whereas the absence of w^- males implied that the X chromosome carried a lethal mutation that was rescued by the presence of *Dp(1;2)51b*. Lines lacking w^- males were then crossed to dm^1-carrying females to test for non-complementation. Two of the newly-induced lethal mutations proved to be allelic to dm^1. The trans-heterozygous females were small with slender bristles and were sterile. These alleles were named dm^2 and dm^3.

To sequence these alleles, stocks were generated in which the lethal *dm* allele was carried in trans to an FM7 balancer carrying a *Krüppel-Gal4, UAS-Green Fluorescent Protein* (*Kr-GFP*) reporter gene (Casso et al., 2000). Mutant larvae, identified by their lack of fluorescence, were collected and DNA was prepared from them. We then used PCR to amplify the *dm* coding regions, and the DNA products of these reactions were sequenced directly.

Generation of germline and follicle cell mitotic clones

Homozygous *dm* mutant germline and follicle cell clones were generated by FLP/FRT-mediated site-specific recombination (Duffy et al., 1998; Xu and Rubin, 1993). Mosaic females carried a *dm* mutation and the 18D FRT in trans to a chromosome carrying the 18D FRT and a hGFP reporter gene (Clarkson and Saint, 1999). They carried FLPase on the second chromosome, either under the control of the heat shock promoter (*hs-FLP*), or expressed from a *UAS-FLP* construct by the somatically-expressed *e22c-Gal4* enhancer trap insertion (Duffy et al., 1998). Females carrying *hs-FLP* were heat shocked for 1 hour at 37°C twice daily for two days. Following heat shock, females were fed on yeast for 2-10 days, and their ovaries were dissected and examined by immunohistochemistry. *dm* mutant cells were identified by the absence of GFP expression. Staging of egg chambers was carried out according to Spradling (Spradling, 1993).

Immunohistochemistry and BrdU labeling

Immunohistochemistry was carried out as described previously (Niewiadomska et al., 1999), with the following modifications. For immunostaining, ovaries were fixed for 15 minutes in 5% paraformaldehyde in PBS plus heptane. Prior to staining, ovaries were blocked by incubation in PBS with 0.1% Tween-20 and 1% BSA. The following primary antibodies were used: mouse monoclonal anti-Fasciclin III (1:50 primary dilution) (Patel et al., 1987), provided by the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa; rabbit anti-Dacapo (1:500 primary dilution), provided by Christian Lehner (Lane et al., 1996); mouse monoclonal anti-Cyclin E (1:5), provided by Helena Richardson (Richardson et al., 1995); rabbit anti-ORC2 (1:2500), provided by Stephen Bell (Royzman et al., 1999); anti-Broad Complex (1:100), from Greg Guild (Emery et al., 1994); rabbit anti-phosphohistone H3 (1:500) (Upstate Biotechnology); and rabbit anti-dMyc (1:5000). Antisera against dMyc protein was generated in rabbits following the injection of a histidine-tagged fusion protein encoding amino acids 80-322 of dMyc. Rhodamine or Alexa 594-conjugated secondary antibodies were used at a dilution of 1:500 (Molecular Probes), for the detection of all primary antibodies.

BrdU labeling was carried out as described (Calvi et al., 1998), with the following modifications. Dissected ovaries were incubated in 1

mg/ml BrdU (Sigma) in Grace's Medium (Mediatech) for 1 hour at room temperature, followed by fixation in 5% paraformaldehyde in PBS plus heptane for 10 minutes. Fixed ovaries were then incubated for 1 hour in PBS with 0.6% Triton X-100, followed by incubation in DNase buffer [66 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol] with 100 U DNase I (Roche). BrdU was detected by a mouse anti-BrdU antibody (Becton Dickinson) used at a dilution of 1:50, as described above. Images were captured using a Zeiss Axioplan 2i microscope equipped with a Zeiss Axiocam digital camera and edited for publication using Adobe Photoshop.

For the comparison of nuclear sizes in different genetic backgrounds, nuclear area was determined by pixel density of DAPI-stained nuclei using the UTHSCSA Image Tool for Windows Version 1.28 (University of Texas Health Science Center, San Antonio). GFP images of the same nuclei were used to determine genotype.

Results

Isolation of lethal alleles of *dm*

At the time we initiated our studies of dMyc, the only available mutation was dm^1, a viable allele that produces small flies with slender bristles. Females homozygous for dm^1 are also sterile. Based on its broad expression pattern in *Drosophila* (Gallant et al., 1996; Schreiber-Agus et al., 1997), and its essential role in other species, we presumed that strong mutations in *dm* would be lethal. To isolate such mutations, we employed a strategy that used a duplication that includes the *dm* locus, *Dp(1;2)51b*, to select specifically for mutations in the region covered by the duplication (see Materials and methods). Using this strategy, we isolated two lethal mutations in *dm*, dm^2 and dm^3. Surprisingly, both of the newly-induced mutations resulted from a C→T nucleotide alteration that results in the formation of a stop codon at the normal position of AA 676, leading to truncation of the dMyc protein at AA 675, immediately upstream of the leucine zipper motif (Fig. 1A). Although it is noteworthy that the same mutation was recovered twice, there is no doubt that the two alleles were generated independently. First, the screen described above was carried out twice (the numbers represent the sum of both screens), and only one allele was recovered from each screen. It is also highly unlikely that the two alleles were inadvertently mixed, because the chromosome carrying the dm^2 allele also carried a second mutation at the *vermillion* locus, not present on the dm^3-carrying chromosome. The phenotypic effects of the two alleles on fly viability, and on the growth of ovarian follicle and nurse cells were indistinguishable. The results of our studies of dm^2 are reported here.

Based on the nature of the dm^2 mutation, the dm^2 allele would be expected to express protein, which we have confirmed by antibody staining (data not shown). However, according to results obtained in studies of Myc (MacGregor et al., 1996; Prendergast and Ziff, 1991), we would expect the truncated protein produced by the dm^2 to have lost the ability to interact with Max. To determine the severity of dMyc loss-of-function associated with the dm^2 mutations, we generated females in which the hypomorphic dm^1 allele was carried in trans to dm^2 (and dm^3) and compared these females to those in which dm^1 was carried in trans to a deficiency uncovering *dm.* In comparisons of body and bristle sizes, and of ovary phenotypes, dm^1/dm^2 and dm^1/dm^3 females were indistinguishable from dm^1/Df females (data not shown). Although we cannot rule out the possibility that the dm^2-encoded protein retains some residual activity, these observations indicate that dm^2 represents a strong loss-of-function allele.

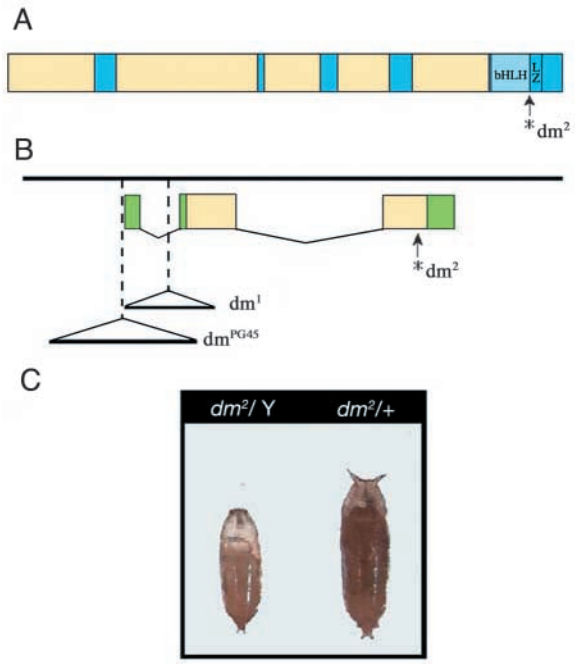

Fig. 1. Structure of the dMyc protein and gene. (A) Segments of the dMyc protein that are similar to stretches of the mouse Myc protein are shown in blue. The leucine zipper (LZ) and basic helix-loop-helix (bHLH) motifs are indicated. The arrow marks the position at which the protein is truncated by the dm^2 mutation. (B) *dm* exons are indicated by boxes, with amino acid coding regions in yellow. The positions of transposon insertions responsible for the dm^1 and dm^{PG45} alleles are indicated, as is the site of the dm^2 point mutation (asterisk). (C) The dm^2/Y pupa (left) is significantly smaller than the dm^2/+ pupa (right).

To determine the lethal period of flies hemizygous for dm^2, stocks carrying dm^2 in trans to an *FM7* balancer expressing a *Kr-GFP* reporter gene were constructed, which enabled dm^2 hemizygous mutant eggs to be identified by their lack of fluorescence. Although dm^2 eggs hatched at near normal rates, and many mutant larvae were viable for several days following hatching, they remained small and only about 20% of mutant larvae progressed to pupation. The few pupae that formed were extremely small relative to wild type (Fig. 1C), and none of the mutant pupae eclosed as adult flies.

To investigate whether embryonic development requires maternal loading of dMyc mRNA, we used the dominant female sterile technique (Chou and Perrimon, 1996) to generate germline clones of dm^2. However, we found that oogenesis in these females arrested relatively early and they did not lay eggs (data not shown). This indicated that *dm* expression is required in the germline for the progression of oogenesis. Consequently, we were not able to determine whether maternally-expressed dMyc is required for embryonic development.

dMyc is required cell-autonomously in the female germline for growth and endoreplication, but not for cell division

Antibody staining revealed that dMyc protein is abundantly

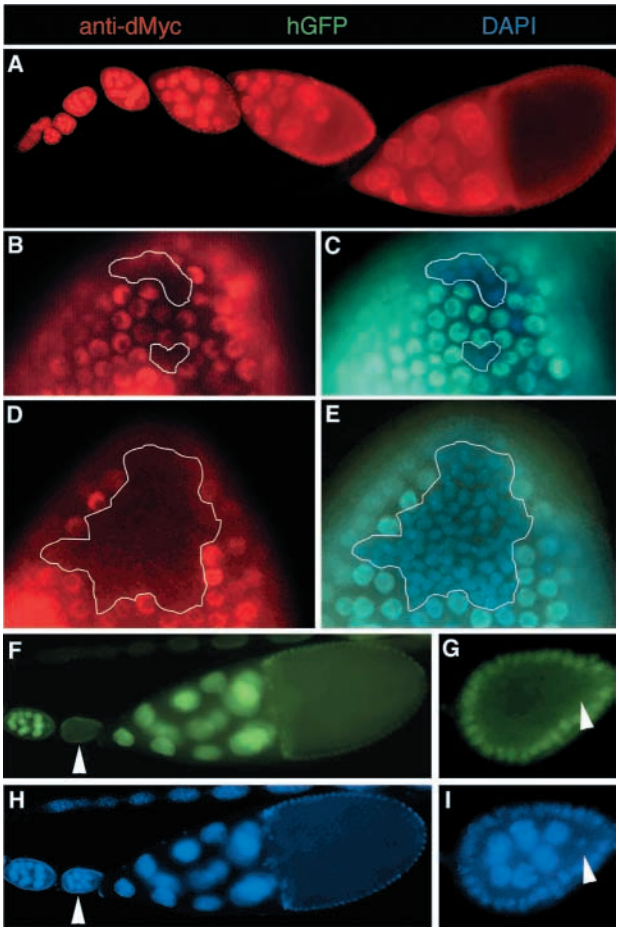

Fig. 2. Expression of dMyc in the ovary, and morphology of *dm*^{2} germline clones. (A) In wild-type ovaries, dMyc is present in the nuclei of germline and somatic cells at most stages of oogenesis. (B,C) Nuclear dMyc is absent from follicle cells homozygous for *dm*^{1} (white outlines), identified by their blue color in the hGFP/DAPI overlay. (D,E) Nuclear dMyc is also absent from follicle cells homozygous for *dm*^{PG45} (white outlines; identified by their blue color in the hGFP/DAPI overlay). (F,H) Egg chambers with a *dm*^{2} mutant germline (arrowhead), identified by the absence of hGFP, appear smaller than normal, on the basis of their position in the ovariole. (G,I) Oocyte specification is not apparently affected in *dm*^{2} mutant germ cells. The oocyte nucleus labels faintly with DAPI (arrowhead), and is positioned at the posterior of this stage 4 egg chamber.

expressed, and localized to the nuclei of both nurse cells and follicle cells during oogenesis (Fig. 2A). To demonstrate the specificity of the antibodies for dMyc, we generated homozygous-mutant follicle cell clones of *dm*^{1}, which has previously been shown to cause a reduction in the expression of *dm* RNA in the ovary (Gallant et al., 1996). Consistent with this, *dm*^{1}/*dm*^{1} mutant clones exhibited a strong reduction of staining compared with their wild-type neighbors (Fig. 2B,C). Antibody staining was also lost in follicle cells clones homozygous for *dm*^{PG45} (Fig. 2D,E), another lethal allele of *dm* that has recently been identified (Bourbon et al., 2002), which results from a transposon insertion in the locus.

To further investigate the germline requirement for dMyc function during oogenesis, we generated mosaic females in which *dm*^{2} homozygous mutant germline cells were identifed by their lack of hGFP expression. During normal oogenesis, a single germline stem cell divides asymmetrically to produce another stem cell and a cystoblast that is destined to differentiate (Spradling, 1993). The cystoblast divides mitotically four times with incomplete cytokinesis to produce 16 interconnected germ cells: the oocyte and 15 nurse cells. Using the FLP/FRT technique, we generated females in which all the germline cells were homozygous for *dm*^{2} (Fig. 2F-I). The recovery of follicles in which all 16 germline cells were mutant for *dm*^{2} indicates that at least four cycles of mitotic division occurred following the loss of the wild-type *dm* gene. In addition, *dm*^{2} mutant germline clones also appeared to allow normal oocyte differentiation. The oocyte can be identified at the posterior of the egg chamber by its smaller nucleus, which contains reduced amounts of DNA relative to its sister nurse cells (Fig. 2G,I). In addition, the localization of oocyte-specific RNAs, such as *orb*, was detectable and restricted to a single cell in the mutant germline cysts (data not shown).

Table 1. BrdU incorporation rates in germline and follicle cell *dm*^{2} clones

	Genotype	
	FRT18D/FRT18D	*dm*^{2} 18D/*dm*^{2} 18D
Germline clones stage 5-8	28% (*n*=630 cells)	10% (*n*=435 cells)
Mosaic germline clones stage 5-8	31% (*n*=136 cells)	6% (*n*=136 cells)
Follicle cell clones stage 8-10	37% (*n*=410 cells)	12% (*n*=449 cells)

Ovaries containing germline or follicle cell clones were incubated with BrdU for one hour. BrdU incorporation was detected using an anti-BrdU antibody. Frequency of BrdU incorporation in wild-type clones (FRT18D) is compared with *dm*^{2} clones (*dm*^{2} FRT 18D) of approximately the same stage. Results are expressed as the percentage of BrdU-positive cells per total number of clonal cells.

The most prominent feature of the *dm*^{2} mutant germline clones was the failure of the nurse cell nuclei to undergo their normal dramatic increase in size, suggesting a defect in growth or endoreplication. Following the completion of the mitotic divisions that form the 16-cell germline cyst, the nurse cells undergo a period of rapid cell growth and endoreplication, in which DNA is replicated in the absence of cell division. To assess the ability of the *dm*^{2} mutant cells to endoreplicate, we measured bromo-deoxyuridine (BrdU) incorporation in stage 5-8 egg chambers containing germline clones. Because nurse cells become post-mitotic prior to stage 3 of oogenesis, incorporation of BrdU in stage 5-8 egg chambers is a direct measure of endoreplication-associated DNA synthesis. In egg chambers with germline clones of a control chromosome, 28% of the cells were seen to have incorporated BrdU during the one hour labeling period, whereas only 10% of the *dm*^{2} mutant cells had (Table 1). This result indicates that dMyc function is required for germline cells to carry out the endoreplication cycles required for normal oogenesis.

During the course of these studies, we fortuitously identified mosaic egg chambers in which the FLP/FRT-mediated recombination event occurred subsequent to the stem cell/cystoblast division, resulting in germline cysts that contained both wild-type and mutant cells (Fig. 3). Nuclei from nurse cells mutant for *dm*^{2} were dramatically reduced in size compared with the wild-type nuclei in the same cyst (Fig.

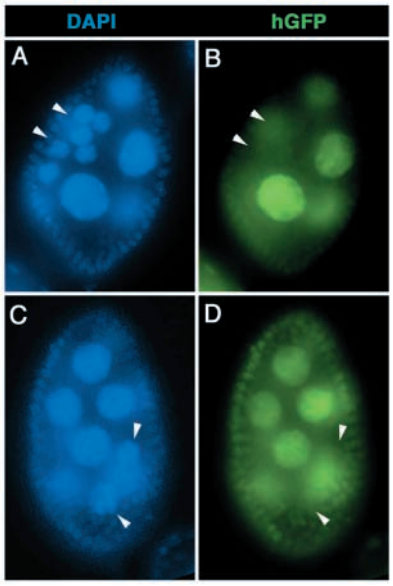

Fig. 3. Homozygous dm^2/dm^2 nurse cells present in mosaic egg chambers exhibit cell-autonomous growth defects. (A,B) The nuclei of dm^2/dm^2 mutant germ cells, which stain with DAPI but do not exhibit hGFP-associated fluorescence (arrowheads), are significantly smaller than the nuclei of their wild-type sister nurse cells. (C,D) dm^2 mutant nurse cell nuclei, visualized with DAPI (arrowheads), often exhibit a blob-like morphology indicating the presence of polytene chromosomes, in the same egg chambers as wild-type nuclei exhibiting the more dispersed polyploid DNA morphology, characteristic of relatively more mature nurse cells.

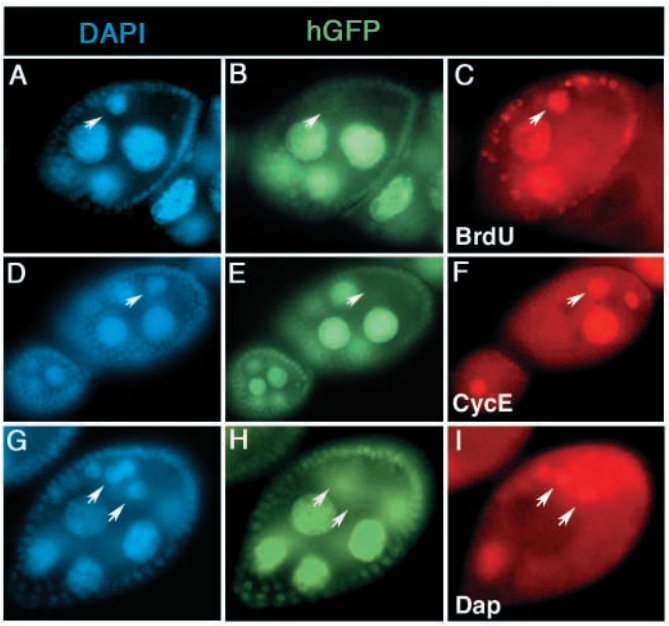

Fig. 4. dm^2 mutant nurse cell nuclei undergo DNA endoreplication. (A-C) dm^2 mutant nuclei (arrowhead) incorporate BrdU. (D-F) dm^2 mutant germ cells (arrowhead) express Cyclin E periodically. (G-I) dm^2 mutant germ cells (arrowhead) also express Dacapo periodically.

3A,B). Many of the dm^2 nurse cell nuclei in mixed cysts exhibited morphological characteristics, consistent with reduced levels of endoreplication, that distinguish them from their wild-type sister cells. During the first five rounds of nurse cell DNA endoreplication, nurse cell chromosomes are polytene, with tightly associated chromatids. During these early rounds of nurse cell endoreplication DAPI-stained nuclei exhibit a clearly identifiable morphology [Fig. 3C,D (Dej and Spradling, 1999)], in which condensed chromosomes are apparently visible. However, following the fifth round of DNA endoreplication, nurse cell chromosomes normally exhibit a more dispersed appearance. dm^2 mutant nuclei were delayed in making this morphological transition, so that mutant nuclei exhibiting the condensed chromosomal morphology were often identified in the same egg chambers as wild-type nuclei that displayed the more mature dispersed chromatin phenotype.

To examine the effect of loss of dMyc activity on endoreplication in these mixed germline clones, we measured BrdU incorporation in stage 5-8 mosaic cysts containing eight dm^2/dm^2 and eight wild-type cells (Fig. 4C). We observed that 31% of wild-type nurse cells nuclei took up BrdU, whereas only 6% of dm^2 nurse cell nuclei in the same cysts were labeled (Table 1). This result indicates that wild-type nurse cell nuclei were five times more likely to undergo BrdU incorporation than were dm^2 mutant cells of exactly the same age. Consistent with our observations of a decrease in the size of the nuclei in dm^2 mutant nurse cells, their lowered frequency of BrdU incorporation suggests that dm^2 mutant cells have a reduced capacity to undergo DNA endoreplication. It is notable that in the mixed cysts, the ratio of labeled wild-type cells (31%) to labeled dm^2 mutant cells (6%) was 5:1, whereas in the whole germline clones that ratio was only 2.8:1. This suggests that within a mosaic cyst, competition from the wild-type cells further impairs the ability of the mutant cells to carry out endoreplication.

In addition to egg chambers containing eight dm^2 nurse cells and eight wild-type nurse cells, we also observed mosaic cysts containing two dm^2 and 14 wild-type cells, or four dm^2 and 12 wild-type cells, respectively. Regardless of the number of mutant versus wild-type cells, however, the oocyte appeared to differentiate normally. In addition, there was no apparent bias against the differentiation of a dm^2 mutant oocyte in the mixed germline clones, consistent with our previous finding that the dm^2 mutation does not interfere with oocyte specification.

Absence of dMyc function in the germline is not associated with changes in expression of the cell cycle regulators Cyclin E or Dacapo

Endoreplication in nurse cells is a regulated cell cycle that is thought to be controlled by the relative levels of the G1 cyclin, Cyclin E, and by Cyclin dependent kinase 2 (Cdk2) (Edgar and Orr-Weaver, 2001). Cycles of Cyclin E protein expression and breakdown (Lilly and Spradling, 1996) produce cyclical Cdk2 activity, which is believed to be necessary for the phosphorylation of components of the pre-replication complex and the initiation of DNA replication at each endoreplication cycle (Edgar and Orr-Weaver, 2001; Woo and Poon, 2003). To determine whether the reduced ability of dm^2 mutant nurse cells to endoreplicate is due to alterations in Cyclin E expression, we used antibodies against Cyclin E to examine its expression in mosaic germline cysts. In wild-type cells, because Cyclin E is expressed in a cyclical pattern (Lilly and

Spradling, 1996), only a subset of cells is stained by antibodies against Cyclin E at a given time. In mosaic germline cysts, a subset of the cells of each genotype, dm^2/dm^2 and +/+, were positive for Cyclin E staining (Fig. 4F), consistent with a cyclical pattern of expression. Thirty-seven out of 131 (28%) dm^2 nurse cells, and 74 out of 199 (37%) wild-type nurse cells, counted in mosaic germline cysts exhibited Cyclin E expression. The staining observed was of approximately equal intensity in dm^2 versus wild-type nurse cells. Although the basis for the difference in the proportion of dm^2 and wild-type nurse cells expressing Cyclin E is not clear, this result suggests that Cyclin E expression cycles relatively normally in dm^2 mutant cells and that a failure in the periodic expression of Cyclin E is not responsible for the reduced ability of these cells to undergo endoreplication.

The activity of the *Drosophila* Cyclin E/Cdk2 complex is inhibited by Dacapo, the $p27^{cip/kip}$ ortholog (de Nooij et al., 1996; Lane et al., 1996). Like Cyclin E, Dacapo protein levels oscillate in a periodic manner, but slightly out of phase with Cyclin E (de Nooij et al., 2000). To investigate a possible influence of dMyc on Dacapo protein expression, we stained mixed germline cysts with antibodies against Dacapo. Similar to what we observed for Cyclin E, Dacapo protein was detected in a subset of both dm^2 and wild-type nurse cells (Fig. 4I), consistent with an oscillating pattern of protein expression. This indicates that the reduced endoreplication seen in dm^2 mutant nurse cells does not arise through alterations in the expression of Dacapo protein. These results, together with the ones described above, also indicate that, in these cells, dMyc is not directly required for the expression of either Dacapo or Cyclin E.

Taken together, the studies described above demonstrate that dMyc activity is not required in the germline for the mitotic divisions that generate the 16-cell cyst, nor for the specification of the oocyte. By contrast, dMyc plays an essential role during the stages of oogenesis in which growth and DNA endoreplication occur in the nurse cells. Although dm^2 mutant nurse cells are capable of undergoing the transition from the mitotic cycle to endoreplication, the number of endocycles that occur in these cells is dramatically reduced relative to wild-type nurse cells.

Follicle cell growth and endoreplication requires dMyc function

Endoreplication is also carried out by the somatically-derived follicle cells that surround the germline cells during oogenesis (Calvi et al., 1998). Follicle cell precursors first associate with the germline cyst shortly after the fourth mitotic division that generates the 16-cell cyst. The somatic cells associated with each cyst divide approximately eight times during stages 2-6, to produce an epithelium of about 1200 follicle cells per egg chamber (Margolis and Spradling, 1995). Upon completion of these mitotic divisions, the follicle cells execute three rounds of endoreplication, which increases their ploidy to 16C (Calvi et al., 1998). Similar to our observations of nurse cells, we find that dMyc is required for efficient DNA endoreplication in follicle cells, but seems not to be essential for their mitotic proliferation. As described for the generation of germline clones, we used FLP/FRT-mediated site-specific recombination to produce marked clones of dm^2/dm^2 mutant follicle cells. We recovered clones containing 10-20 mutant cells or more (Fig. 5A), which indicates that the loss of dMyc activity does not prevent the cells from carrying out several mitotic division cycles. In control experiments we generated marked wild-type clones that contained somewhat larger numbers of cells (data not shown). This observation may reflect a slightly depressed

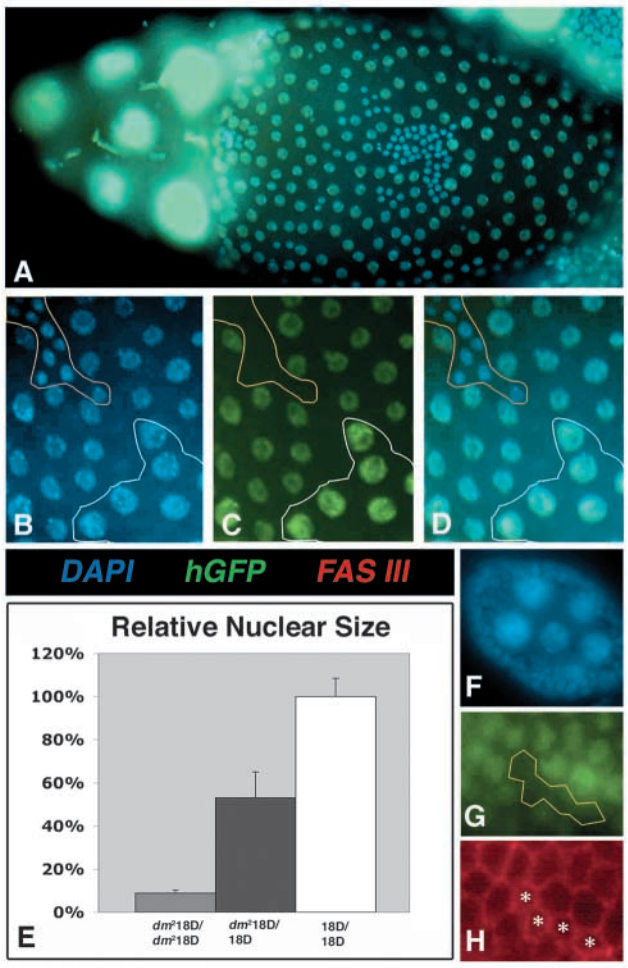

Fig. 5. dMyc is a dose-dependent regulator of follicle cell and nuclear size. (A) An overlay of DAPI/hGFP images shows a large clone of dm^2 follicle cells with nuclei of unusually small size. (B-D) DAPI (B), hGFP (C) and merged (D) images of a field of follicle cells with a clone of dm^2/dm^2 mutants (yellow outline) and twin-spot derived +/+ cells (white outline). The nuclei of +/+ cells (carrying two copies of the hGFP gene and therefore distinguishable from dm^2/+ cells by their increased fluorescent intensity) are larger than the nuclei of their dm^2/+ sister cells, which are larger than the nuclei of the dm^2/dm^2 mutant cells. (E) Measurement of the area of nuclei indicates that homozygous wild-type nuclei are twice the size of dm^2/+ nuclei, and tenfold larger than the nuclei of dm^2/dm^2 mutant cells. See Materials and methods for details on size determination. (F) A DAPI-stained follicle at stage 5, prior to the time at which follicle cell endoreplication initiates. (G) At higher magnification, the lack of hGFP nuclear staining (white outline) indicates the position of a clone of dm^2/dm^2 mutant follicle cells. (H) Anti-Fas III staining, which weakly stains the periphery of all follicle cells, demonstrates that the homozygous mutant cells (asterisks) are considerably smaller than the neighboring follicle cells. Thus, dMyc regulates follicle cell growth prior to the onset of endoreplication.

proliferative ability of the dm^2/dm^2 cells, compared with $dm^2/+$ cells, attributable to their decreased growth rates. A similar situation has been observed in *dm* mutant clones generated in other tissues (Johnston et al., 1999).

To assess the ability of dm^2 mutant follicle cells to undergo endoreplication, we compared the frequency of BrdU incorporation in cells contained within dm^2 mutant clones to that of cells contained within control clones, homozygous for an otherwise wild-type *FRT18D*-carrying chromosome (Table 1). The percentage of cells that incorporated BrdU was scored in stage 8-10 egg chambers, in which the follicle cells are post-mitotic and BrdU incorporation is thus a measure of DNA synthesis occurring in the endocycle. To confirm that *dm* mutant cells in stage 8-10 follicles were indeed post-mitotic, we stained mosaic follicles with an antibody against phosphohistone H3 (PH3), a marker for mitotically active cells. Like their wild-type counterparts, dm^2 mutant cells did not express the PH3 antigen at this stage of oogenesis (data not shown). We found that 37% of the wild-type follicle cells contained within control clones stained for BrdU incorporation, whereas only 12% of dm^2/dm^2 follicle cells were observed to have incorporated BrdU under the same conditions (Table 1, Fig. 6B). Although the proportion of *dm* cells that incorporated BrdU was lower than that of wild-type cells, the intensity of the BrdU staining in the nuclei of *dm* mutant cells was approximately equal to that seen in the nuclei of the *dm/+* cells outside the clone (data not shown). Taken together, these results indicate that follicle cells lacking dMyc function undergo some DNA endoreplication, but at reduced frequencies relative to wild-type cells. Based on the size of the nuclei of dm^2 mutant cells (see below), we estimate that dm^2 follicle cells are unlikely to complete more than one cycle of DNA endoreplication.

The nuclear size of dm^2/dm^2 homozygous mutant cells in egg chambers that progressed beyond stage 9 was an order of magnitude smaller than +/+ cells present in the same epithelium (Fig. 5A,E). Strikingly, we also observed a 50% decrease in the nuclear size of $dm^2/+$ heterozygous cells relative to their +/+ neighbors (Fig. 5B-E), which demonstrates that dMyc exerts a dose-dependent effect on nuclear size in follicle cells. Similarly staged egg chambers were also stained with an antibody against the cell surface protein Fasciclin III (Fas III), in order to assess the size of dm^2 mutant follicle cells. Like their nuclei, dm^2 mutant follicle cells were invariably smaller than wild-type follicle cells present in the same follicle layer (data not shown).

Cell growth and endoreplication are intimately linked cellular processes (Edgar and Orr-Weaver, 2001). Thus, a failure to grow can lead to impaired endoreplication, and vice versa. In the germline, endoreplication begins shortly after the formation of the 16-cell cyst, making it difficult to ascertain whether the effect of the dm^2 mutation on endoreplication is a direct or indirect result of reduced growth. In the follicle cell layer, however, we detected a decrease in the size of dm^2 mutant follicle cells relative to wild-type cells at stage 5, prior to the time at which they begin endoreplication (Fig. 5G,H). This finding indicates that the loss of dMyc function directly affects the ability of the follicle cells to grow, with the effects on endoreplication potentially being a secondary consequence of this effect.

Chorion gene amplification does not require dMyc function and is independent of endoreplication

Late in oogenesis, the follicle cells are responsible for the secretion of the eggshell, which is composed of the vitelline membrane, the endochorion and the exochorion. To facilitate the synthesis of large amounts of chorion proteins, the follicle cells specifically amplify two clusters of chorion genes, located on the X and the third chromosomes, as well as two other loci that have not yet been identified (Calvi et al., 1998). Although the precise mechanisms that regulate chorion gene amplification have not been elucidated, Cyclin E activity is known to be required both to promote amplification of the appropriate gene clusters, and to prevent the amplification of other genes elsewhere on the chromosomes (Calvi et al., 1998).

Chorion gene amplification can be visualized through BrdU incorporation, and appears as small and discrete foci that stain with antibodies against BrdU. In stage 11 mosaic egg chambers in which we assessed BrdU incorporation, we observed that BrdU-staining puncta were present not only in wild-type follicle cells, but also in dm^2/dm^2 homozygous mutant cells (Fig. 6D). To obtain supporting evidence that these puncta represented sites of gene amplification, we stained mosaic follicles with an antibody directed against a component of the origin recognition complex (ORC2), which has been shown to be associated with chorion clusters that are undergoing amplification (Royzman et al., 1999). Like BrdU, discrete foci of ORC2 staining were observed in both wild-type and dm^2/dm^2 mutant follicle cells (data not shown). These observations indicate that, despite the inability of dm^2 mutant follicle cells to undergo a normal course of DNA endoreplication, they are nevertheless able to carry out gene amplification. To our knowledge, *dm* represents the first identified mutation that perturbs the follicle cell endocycle without eliminating gene amplification.

One of the loci thought to be required for chorion gene amplification is the *Broad-Complex* (*BR-C*; *broad* – Flybase), an early ecdysone-response gene that acts at many times in the fly life cycle and which encodes a family of zinc-finger transcription factors. A mutation in the *BR-C* locus causes premature arrest of chorion gene amplification, whereas overexpression of *BR-C* isoforms leads to the formation of additional foci of BrdU incorporation in follicle cells that presumably represent inappropriate sites of gene amplification (Buszczak et al., 1999). Consistent with their apparent ability to carry out chorion gene amplification, we found that dm^2/dm^2 mutant follicle cells exhibited normal BR-C protein expression (data not shown).

Loss of dMyc activity in either the germline or the follicle cell layer severely delays the maturation of the entire egg chamber

In addition to the cell-autonomous effects of the loss of dMyc on dm^2 mutant cells, we found that when either the germline or the follicle cell layer lacked dMyc function the maturation of the entire follicle was affected. Egg chambers in which all germline cells were homozygous for dm^2 remained small and appeared to be immature relative to their age (Fig. 7A,B). Follicles that, on the basis of their position in the ovariole, should be between stages 8-10, instead had the appearance of stage 6-7 follicles. The wild-type follicle cells that surrounded the mutant germline exhibited unusual patterns of gene

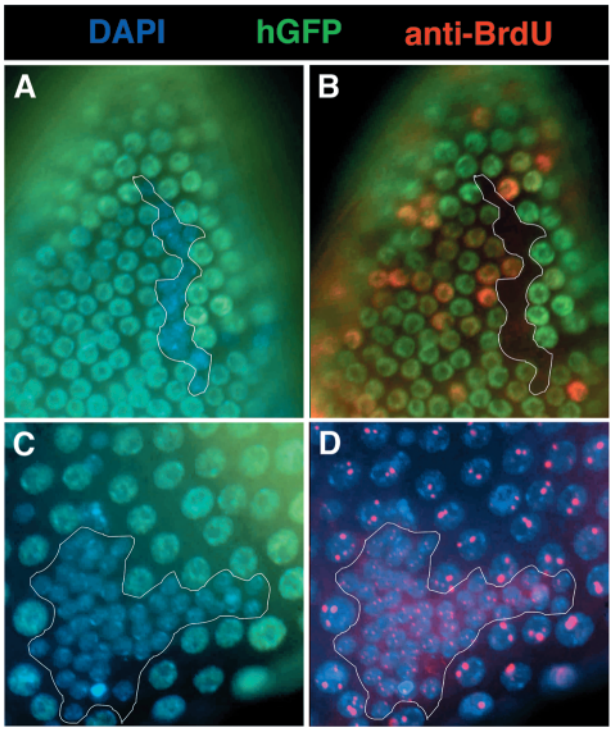

Fig. 6. Follicle cell DNA endoreplication, but not gene amplification, is perturbed by mutations in *dm*. (A) An overlay of DAPI/hGFP images shows a clone of homozygous dm^2/dm^2 mutant cells outlined in white. (B) An overlay of hGFP/anti-BrdU images of the same field of follicle cells shows that a much smaller proportion of dm^2 mutant follicle cells than wild-type cells incorporate BrdU. (C) An overlay of DAPI/hGFP images shows a large clone of dm^2/dm^2 mutant cells, present in a stage 11 egg chamber, outlined in white. (D) In the overlay of DAPI/BrdU images of the same mutant clone, gene amplification can be detected by the presence of puncta of BrdU incorporation in both the heterozygous and dm^2 mutant cells.

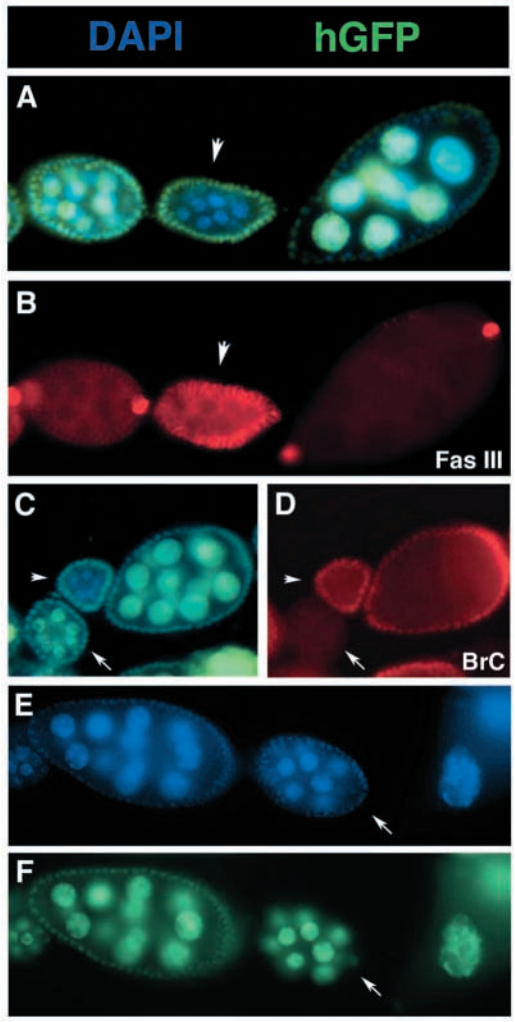

Fig. 7. Growth of germline and somatic tissues in the ovary is tightly coordinated. (A,B) An egg chamber with a dm^2 mutant germline (arrowhead) exhibits abnormal perdurance of Fas III expression in the overlying wild-type follicle cells. (C,D) By contrast, an egg chamber with a dm^2 mutant germline (arrowhead) expresses Broad Complex (BR-C) in the follicle cell layer on the basis of its age or position in the ovariole, rather than on its apparent maturity based on size. Note that the adjacent anterior egg chamber (arrow) is larger, but younger, and does not express BR-C. (E,F) An egg chamber in which all follicle cells are mutant for dm^2 (arrow) is abnormally small, with small nurse cells.

expression. For example, Fasciclin III (Fas III), a cell adhesion marker that is expressed by all follicle cells very early in oogenesis, is normally downregulated so that only the two polar cells at each end of the follicle express high levels of Fas III (López-Schier and St Johnston, 2001). In egg chambers in which all the germline cells were mutant for dm^2, the entire follicle cell layer expressed inappropriately high levels of Fas III (Fig. 7B). By contrast, follicle cell expression of BR-C, which is normally initiated during stage 6 in wild-type follicles, was observed to initiate in egg chambers that contain dm^2 mutant germ cells, despite their failure to grow and mature normally (Fig. 7D).

We also recovered egg chambers in which a wild-type germline was surrounded by an epithelium in which every follicle cell was dm^2/dm^2 (Fig. 7E,F). These large somatic clones resulted from recombination events that occurred in the stem cell precursors to the follicle cells. These egg chambers were severely delayed in their growth and maturation, and rarely progressed to a stage in which yolk uptake could be detected in the oocyte. Taken together, these results indicate that the failure of either the germline or the somatic component of the follicle to grow normally prevents the progression through oogenesis of the complementary somatic or germline cells, respectively. Although these findings do not necessarily indicate a direct role for dMyc in the communication of developmental signals between the germline and the soma, they do imply the existence of a regulatory mechanism that coordinates the growth and development of the two components.

Discussion

In the work reported here, we have used a newly-isolated lethal allele to study the role of dMyc in *Drosophila* oogenesis, a process that involves cell proliferation, differentiation and growth. Our results indicate that the primary consequence of the loss of dMyc activity from either nurse cells or follicle cells

is a reduced ability to grow and support DNA endoreplication. Our results are consistent with a previous report that loss of dMyc function in the developing wing disc retards cellular growth and reduces final cell size (Johnston et al., 1999). dMyc is also required for endoreplication and growth of larval cells (S. Pierce and R. Eisenman, personal communication).

The phenotype of dm^2 mutant cells in the ovary is consistent with a requirement for dMyc function in both cell growth and the endocycle, two processes which are interdependent. Conditions that block cell growth invariably perturb endocycle progression (Galloni and Edgar, 1999). Conversely, the inhibition of DNA synthesis during the endocyle, through the expression of inhibitors of DNA replication or by mutating genes essential for DNA replication, leads not only to a slowed increase in DNA content but also to a decrease in overall cell growth (reviewed by Edgar and Orr-Weaver, 2001). We favor the idea that dMyc activity is required initially to enhance growth, with the effects of dMyc loss-of-function on endoreplication being a secondary consequence of impaired growth. At oogenic stages prior to the onset of endoreplication in the follicular epithelium, dm^2 mutant follicle cells are detectably smaller than their wild-type neighbors. This indicates that reduced dMyc function affects cell growth prior to, and independently of, its effect on endoreplication. Because of the reciprocal relationship between growth and endoreplication, the initial growth defect in dm^2 cells may lead to endoreplication defects that then feed back and contribute to further reductions in cell growth. Ultimately this growth defect could have the effect of greatly reducing the number of endoreplication cycles that dm^2 mutant cells can complete. However, we cannot rule out a direct effect of dMyc on endoreplication, in addition to its influence on growth.

Although endoreplication is severely impaired when dMyc activity is reduced, *dm* mutant cells maintain a limited ability to endocycle. During a one-hour pulse of BrdU, dm^2 mutant germline cells were 3- to 5-fold less likely than wild-type cells to be in the S phase of the endocycle, as measured by BrdU incorporation. This suggests that relative to wild-type cells, dm^2 mutant cells spend a longer fraction of the endocycle in G1 phase and a shorter proportion in S phase. A simple explanation for this observation would be that endoreplicating cells remain in G1 until they reach a critical metabolic or growth threshold required for the onset of DNA synthesis, and the rate at which they reach this threshold depends on the level of dMyc activity. This implies that an important function of dMyc in the ovary is to promote growth during G1 so that the G1/S progression can occur. This interpretation would be consistent with the observation by Johnston et al. (Johnston et al., 1999) that overexpressing dMyc in wing disc cells decreases the proportion of the cell cycle spent in G1.

In contrast to its dramatic effects on cell growth and endoreplication, dMyc appears to be largely dispensable for the mitotic proliferation of both germline and somatic cells. Not only did we recover germline and follicle cell clones that resulted from mitotic recombination subsequent to egg chamber formation, we also identified clones that had been produced during the division of the stem cell precursors of these two cell types. This indicates that ovarian cells are capable of dividing many times in the absence of wild-type dMyc activity, which strongly argues against a role for dMyc in mitotic cell cycles in the ovary. Although we favor the notion that mitotic proliferation of these cells can occur in the total absence of dMyc activity, we cannot currently rule out the possibility that the mitotic proliferation that we observe is supported by residual activity associated with the truncated protein produced by the dm^2 mutant allele.

In contrast to our observations, investigations carried out in mammalian tissue culture have suggested a crucial role for Myc in cell cycle progression. Exposure of quiescent cells to growth factors rapidly induces the expression of Myc (Kelly et al., 1983), and forced expression of Myc in various cell types can induce them to enter S phase (Eilers et al., 1991), accelerate their rate of cell division (Karn et al., 1989), and alter their requirements for growth factor stimulation (Stern et al., 1986). Correspondingly, reduction of *Myc* expression is correlated with exit from the mitotic cycle and cell differentiation (Heikkila et al., 1987; Hurlin et al., 1995). In the developing *Drosophila* wing disc, overexpression of dMyc dramatically shortened the length of G1, but a concomitant increase in the length of S phase and G2 resulted in no change in the length of the cell cycle overall (Johnston et al., 1999). Thus, in *Drosophila*, the primary function of dMyc may be to promote cell growth rather than cell proliferation.

Overexpression of dMyc has been proposed to accelerate the G1/S transition in wing disc cells by activating Cyclin E through a post-transcriptional mechanism (Johnston et al., 1999; Prober and Edgar, 2000). Because Cyclin E has been identified as an important regulator of the endocycle in nurse cells (Duronio and O'Farrell, 1995; Knoblich et al., 1994; Lilly and Spradling, 1996), we examined the protein expression of both Cyclin E and Dacapo, the *Drosophila* p27 Cip/Kip homolog that specifically inhibits Cyclin E/Cdk2 activity (de Nooij et al., 1996; Lane et al., 1996). Both Cyclin E and Dacapo protein expression continued to cycle in dm^2 mutant nurse cells. These observations suggest that the effect of the dm^2 mutation on the endocycle does not result from an influence on the pattern of Cyclin E expression. Consistent with this conclusion, Cyclin E protein levels also continue to oscillate in larval fat body and salivary gland cells that overexpress dMyc (S. Pierce and R. Eisenman, personal communication). Perturbations in Cyclin E expression can also lead to the differentiation of multiple oocytes in a single cyst (Lilly and Spradling, 1996). Our finding that only one oocyte differentiated in dm^2 mutant cysts provides further evidence that Cyclin E regulation was relatively normal.

In mammalian cells, expression of the Dacapo homologs p21CIP1 and p27KIP1 are repressed by Myc (reviewed by Gartel and Shchors, 2003). In the *Drosophila* ovary, Dacapo present in the oocyte nucleus prevents it from undergoing DNA endoreplication, helping to maintain it in prophase I of meiosis (Hong et al., 2003). These findings suggested a possible mechanism whereby dMyc-mediated inhibition of *dacapo* gene expression in nurse cells might facilitate their endoreplication. However, the fact that Dacapo protein did not show an obvious increase in expression in dm^2 mutant germ cells suggests that in contrast to what is seen in mammals, *dacapo* expression is not directly influenced by dMyc in these cells, nor is dMyc-mediated repression of *dacapo* expression an important mechanism regulating nurse cell endoreplication.

In *Drosophila*, cell growth is known to be regulated by two distinct but interacting signalling pathways: one mediated through the insulin receptor (InR) and phosphatidylinositol-3-

OH kinase (PI3K), and the other through the nutrition-sensing protein kinase TOR (target of rapamycin) (reviewed by Johnston and Gallant, 2002; Oldham and Hafen, 2003). The *dm* mutant phenotype closely resembles that of mutants affecting the dTOR effector protein ribosomal S6 kinase (Perrimon et al., 1996; Montagne et al., 1999). In mammals, S6 kinases have been shown to promote the translation of ribosomal proteins and translation factors (Jefferies et al., 1997). In a recent study that examined the binding to DNA of the *Drosophila* Myc network proteins Myc, Max and Mad/Mnt, a number of genes involved in ribosome biogenesis and proteins synthesis were identified as dMyc targets (Orian et al., 2003). Taken together, these findings suggest that dMyc, like dS6K, may exert its effect on growth through the enhancement of protein translation.

One protein through which dMyc might exert its effects on translation is the product of the *pitchoune (pit)* gene, a putative DEAD-box RNA helicase (Zaffran et al., 1998) whose human homolog, MrDB, has been shown to be a transcriptional target of Myc-Max heterodimers (Grandori et al., 1996). *pit* mutants exhibit a constellation of phenotypes similar to that observed for dm^2 mutants, and constitutive expression of dMyc induces expression of *pit* in embryos (Zaffran et al., 1998) and third instar larvae (Orian et al., 2003). The identity of Pitchoune protein as a DEAD-box RNA helicase and its subcellular localization in the nucleolus suggests that it may be involved in rRNA processing or ribosome biogenesis (Zaffran et al., 1998).

Perturbation of Delta/Notch signaling between the germline and the follicle cells has been observed to disrupt follicle cell endoreplication (Deng et al., 2001; López-Schier and St Johnston, 2001). Follicle cells homozygous for loss-of-function alleles of *Notch* exhibit a delay in exiting the mitotic division cycle, which leads to overproliferation of the follicular epithelium and the formation of abnormally small mutant cells with small nuclei. This phenotype has been interpreted as an inability of the *Notch* mutant cells to undergo their normal program of differentiation (López-Schier and St Johnston, 2001). By contrast, our observations suggest that *dm* mutant follicle cells exhibit reduced levels of postmitotic DNA synthesis not because they cannot make the transition from mitosis to the endocycle, but because they are unable to grow sufficiently well to support the endocycle. In addition, the execution of chorion gene amplification by dm^2 follicle cells suggests that they adopt at least some of the characteristics of mature follicle cells. This observation may reflect the possibility that dMyc regulates distinct effectors of endocycle DNA replication that do not participate in gene amplification. Alternatively, the metabolic and synthetic needs of cells undergoing gene amplification may be much lower than those of endocycling cells, and may not require the concerted action of the dMyc-activated gene network.

In addition to the effects of loss of dMyc activity on the growth of homozygous mutant germline or follicle cells, we also observed a profound effect on the growth and development of the entire egg chamber when either the complete germline or the entire follicular epithelium was mutant for dm^2. In both cases, the follicles were delayed in their development and rarely progressed to vitellogenic stages, in which yolk uptake can be detected in the oocyte. These results suggest that the growth of the somatic and germline components of the ovary are tightly coordinated. In addition to their failure to grow, the genotypically wild-type follicle cells surrounding dm^2 mutant germline clones exhibited some signs of immaturity, such as perdurance of uniform FasIII expression. Surprisingly, expression of the BR-C by these cells appeared to be determined by their age, based upon their position within the ovariole, rather than on the maturity of the egg chamber, as judged by its size. This raises the interesting possibility that follicle cells can assess their age by a mechanism that is independent of the growth state of the egg chamber in which they are contained. Alternatively, the ability of the follicular epithelium to respond to ecdysone signaling may depend in part on influences external to an individual follicle. It has been well documented that signaling between the soma and the germline is required for the establishment of the dorsoventral and anteroposterior axes of both the follicle and the future embryo (Gonzalez-Reyes et al., 1995; Roth et al., 1995). However, the signals that communicate the growth status of one tissue to the other are less well understood. Our finding that the loss of dMyc function in either the germline or in the soma is sufficient to prevent the growth of the complementary soma or germline, respectively, may provide a useful tool for investigating how this information is transferred between the two tissues

In contrast to clones comprising entire germline cysts, the loss of dMyc activity from a subset of nurse cells led to an apparently autonomous deficiency in growth that did not affect the wild-type nurse cells present in the same cyst. The recovery of such mixed-phenotype mosaic cysts also permitted us to conclude that the reduced growth and impaired endoreplication detected in dm^2 mutant nurse cells did not result from the failure of a general cyst-wide developmental transition that requires the function of dMyc. Indeed, the ability to generate such mixed phenotype cysts was quite surprising, as the nurse cells are interconnected by cytoplasmic bridges and are typically considered to share a common cytoplasm. The restriction of hGFP to the nurse cells in which it was synthesized, and the phenotypic differences between the dm^2/dm^2 and +/+ nurse cells in the same cyst, convincingly demonstrate that there are restrictions that limit the intercellular movement of at least some gene products between nurse cells. The use of the hGFP marker will make it possible to investigate whether the products of other genes expressed by the nurse cells are similarly confined to their cells of origin.

In summary, our analysis of the function of dMyc in the *Drosophila* ovary is consistent with the conclusions of other recent work that indicate that Myc family proteins profoundly influence the ability of cells to grow. By combining the information obtained from genomics-based molecular studies with the genetic analysis of putative target genes, it should be possible to elucidate the role of dMyc in different tissues, and to identify those genes that act as its downstream effectors. The *dm* ovarian phenotype will provide a useful framework in which to investigate the function of the dMyc network in growth and endoreplication.

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