

Drosophila Myt1 is a Cdk1 inhibitory kinase that regulates multiple aspects of cell cycle behavior during gametogenesis

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Summary

The metazoan Wee1-like kinases Wee1 and Myt1 regulate the essential mitotic regulator Cdk1 by inhibitory phosphorylation. This regulatory mechanism, which prevents Cdk1 from triggering premature mitotic events, is also induced during the DNA damage response and used to coordinate cell proliferation with crucial developmental events. Despite the previously demonstrated role for Myt1 regulation of Cdk1 during meiosis, relatively little is known of how Myt1 functions at other developmental stages. To address this issue, we have undertaken a functional analysis of *Drosophila* Myt1 that has revealed novel developmental roles for this conserved cell cycle regulator during gametogenesis. Notably, more proliferating cells were observed in *myt1* mutant testes and ovaries than controls. This can partly be attributed to ectopic division of

germline-associated somatic cells in *myt1* mutants, suggesting that Myt1 serves a role in regulating exit from the cell cycle. Moreover, mitotic index measurements suggested that germline stem cells proliferate more rapidly, in *myt1* mutant females. In addition, male *myt1* germline cells occasionally undergo an extra mitotic division, resulting in meiotic cysts with twice the normal numbers of cells. Based on these observations, we propose that Myt1 serves unique Cdk1 regulatory functions required for efficient coupling of cell differentiation with cell cycle progression.

Key words: Myt1, Wee1, Cdk1, Cdc25, Meiosis, Mitosis, Spermatogenesis, Oogenesis

Introduction

A conserved molecular mechanism that controls entry into mitosis in eukaryotic cells involves the activation of a cyclin-dependent kinase called Cdk1, which serves as a master regulator of early mitotic events (O'Farrell, 2001). To exit mitosis, Cdk1 activity must then be eliminated by proteolytic degradation of its cyclin subunit. Once mitotic cyclins are re-synthesized during interphase, Cdk1 is maintained in an inactivate state by inhibitory phosphorylation, preventing premature initiation of mitotic events during S phase or when the DNA damage checkpoint is induced, to allow time for DNA repair. This Cdk1 regulatory mechanism is also used to coordinate the timing of mitosis with morphogenetic cell movements, as was first demonstrated by studies of the intricate spatial and temporal pattern of cell division during *Drosophila* gastrulation (Edgar and O'Farrell, 1989; Foe, 1989; Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Ectopic expression and morpholino depletion experiments carried out in *Xenopus* also suggest that Cdk1 regulatory mechanisms involving Wee1 kinases couple cell division with cell movements during gastrulation, implying that this is a conserved developmental mechanism (Leise and Mueller, 2002; Murakami et al., 2004).

Two related classes of Cdk1 inhibitory kinases have been identified in metazoans: Wee1 and Myt1. *Xenopus* and *C. elegans* each contain two Wee1-like kinases and a single Myt1

ortholog, which exhibit distinct expression patterns during development (Lamitina and L'Hernault, 2002; Leise and Mueller, 2002; Murakami et al., 2004; Nakanishi et al., 2000; Okamoto et al., 2002; Wilson et al., 1999). The situation is somewhat simpler in *Drosophila*, which has a single Cdk1 inhibitory kinase of each type: Wee1 and Myt1 (Adams et al., 2000; Campbell et al., 1995; Price et al., 2000). *Drosophila* Wee1 is a nuclear kinase that is essential for regulating Cdk1 during the rapid, maternally controlled S/M nuclear divisions of early embryogenesis; however, Wee1 is otherwise dispensable for zygotic development (Campbell et al., 1995; Price et al., 2000; Stumpff et al., 2004). Myt1 localizes to Golgi and endoplasmic reticulum membranes (Z.J., unpublished), as also reported for the *Xenopus* and human Myt1 orthologs (Booher et al., 1997; Liu et al., 1997; Mueller et al., 1995). The Myt1 kinases were originally characterized as membrane-associated dual-specificity Cdk1 kinases that phosphorylate a threonine (T14) residue of Cdk1, as well as the Y15 site that nuclear Wee1 kinases also target (Booher et al., 1997; Liu et al., 1997; Mueller et al., 1995). These differences in Wee1 and Myt1 protein localization and target site specificity suggest that the metazoan Cdk1 inhibitory kinases have evolved distinct cell cycle regulatory functions required at different stages of development.

Transgenic overexpression and RNAi experiments involving Myt1 suggested that its expression primarily affects the G2 phase of the cell cycle; however, genetic evidence of specific

functions for dMyt1 that are essential for normal development has been lacking (Cornwell et al., 2002; Price et al., 2002). Previously, biochemical studies of the prolonged 'G2-like' growth state of immature oocytes suggest that Myt1 is responsible for inhibitory phosphorylation of Cdk1 during this stage of female meiosis in *Xenopus* (Furuno et al., 2003; Karaïskou et al., 2004; Nakajo et al., 2000; Palmer et al., 1998; Peter et al., 2002) and in the starfish *A. pectinifera* (Okano-Uchida et al., 2003; Okumura et al., 2002). Mutations affecting a *C. elegans* homolog have also implicated Myt1 in the regulation of male meiosis (Lamitina and L'Hernault, 2002). In the present report, we describe the isolation and characterization of mutations affecting *Drosophila* Myt1. Our studies reveal that Myt1 serves regulatory functions that have not previously been described but are important for both mitotic and meiotic cell cycles during gametogenesis. These observations implicate Myt1 in specific Cdk1 regulatory mechanisms that are required for coordinating cell cycle behavior with crucial developmental transitions.

Materials and methods

Generation and identification of Myt1 mutants

The *myt1* locus is at region 64F of the 3rd chromosome, corresponding to the predicted gene *CG10569* (Adams et al., 2000). We used a chromosomal deletion called *Df(3L)64D-F*, predicted to uncover the *myt1* locus and ~25–30 other genes, for our genetic screen (Garcia-Bellido et al., 1994). Before undertaking an F2 genetic screen for mutants uncovered by *Df(3L)64D-F* that could be rescued by a transgenic *myt1* rescue construct, *P{myt1⁺}*, described below, we confirmed that this deletion removed the *myt1* locus by complementation tests with lethal *P*-element alleles of genes flanking the gene (Fig. 1B). Males from an isogenized *p^o e^l* stock were fed a 10% sucrose solution containing 10 mM ethyl methane sulfonate (EMS), then mated with females carrying a third chromosome balancer. The heterozygous F1 male progeny were then crossed to females carrying *Df(3L)64D-F* and the hemizygous non-balancer F2 progeny were scored for morphological defects, lethality and female sterility. To make the *P{myt1⁺}* rescue construct, a 3.6 kb *SacI-EcoRI* fragment, including the entire *myt1* gene and ~1 kb of upstream non-coding DNA, was subcloned from a P1 clone (DS04757) into the *pCaspE* transposon vector (Pirrotta, 1988). Transgenic lines carrying the integrated construct were then generated by standard procedures (Spradling, 1986).

Molecular analysis of the Myt1 mutant alleles

To sequence genomic DNA, single flies of the appropriate genotype were homogenized in a tube with 50 µl buffer (100 mM Tris-Cl pH 7.6, 100 mM EDTA, 100 mM NaCl and 0.5% SDS). Proteinase K was added to a final concentration of 100 µg/ml, and the samples were incubated at 37°C for 30 minutes. Genomic DNA (1 µl) isolated by this procedure was then used as a template for DNA sequencing (Amersham), using oligonucleotide primers positioned at 300–400 bp intervals on both template strands. The sequencing reactions were repeated three times with genomic DNA isolated from different flies of the same genotype, for confirmation.

Phenotypic analysis of the Myt1 mutants and *hs-Cdk1AF* expression experiments

Except as indicated, we used *myt1¹/Df(3L)64D-F* hemizygotes as the representative *myt1* mutants for our phenotypic analysis and their *myt1¹/TM3,Sb* heterozygous siblings, as controls. For testes preparations, 1- or 2-day-old male flies were dissected in 1× PBS or in testis isolation buffer (TIB): 183 mM KCl, 47 mM NaCl, 10 mM Tris (pH 6.8), 1 mM EDTA (Casal et al., 1990) plus 1 mM PMSF,

then rinsed twice with the same buffer. We followed established protocols for the testes and ovariole immunofluorescent localization experiments (Bonaccorsi et al., 2000; Mattheis et al., 2000). The primary antibodies and concentrations used were rabbit anti-PH3 (1/2000; Upstate), rabbit anti-anillin at 1/300 (Field and Alberts, 1995), mouse anti-β-tubulin (1/100; Sigma), mouse anti-BrdU (1/20; Jackson Labs), mouse anti-Hts, clone C17.9C6, obtained from the Developmental Studies Hybridoma Bank (DSHB) at 1/10 (Zaccari and Lipshitz, 1996), rat anti-BamC at 1/2000 (McKearin and Ohlstein, 1995), rabbit anti-Aly at 1/2000 (White-Cooper et al., 2000), rabbit (used at 1/500) and rat (used at 1/1000) anti-Vasa (Lasko and Ashburner, 1990), mouse anti-spectrin obtained from DSHB at 1/200 (Dubreuil et al., 1989), mouse anti-Eya obtained from DSHB at 1/100 (Bonini et al., 1993), rabbit anti-Cnn at 1/500 (Heuer et al., 1995), mouse anti-Mpm2 (1/200, Cell Signaling), and mouse anti-Fas3 obtained from DSHB at 1/5 (Patel et al., 1987). Alexa-488 and Alexa-568 conjugated secondary antibodies (used at 1/1000) were obtained from Molecular Probes, as was rhodamine-conjugated phalloidin. BrdU incorporation was assayed in 1- to 2-day-old dissected testes, using published protocols (Wolff, 2000). For experiments to determine the number of cells per cyst, *w¹¹¹⁸* or *myt1* mutant testes were dissected in TIB containing 8.3 µg/ml Hoechst 33342. Whole cysts were teased out with a bent tungsten needle and imaged using a Zeiss Axioplan 2E epifluorescence microscope, equipped with a black and white Axiocam CCD camera. For the *Cdk1-AF* experiments, 1- to 2-day-old *y w; hs-Cdk1AF* transgenic flies and *y w* controls received 1 hour, 37°C heat shocks twice a day for three consecutive days, prior to dissection.

Genetic tests for segregation defects during female meiosis

As female Myt1 mutants are partially fertile, a genetic cross (cross #1) was set up between *myt1¹/myt1²* virgins (with *myt1¹/TM3, Sb* virgins used in a control cross) and *C (1;Y)I, y, v, f; B; C(4) RM, ci, ey [R]* males to detect non-disjunction (NDJ) events during meiosis. *myt1¹* and *myt1²* were two independently isolated mutant chromosomes with different secondary lethal mutations (allowing us to recover the viable *myt1* mutants). In this cross, non-disjunction of homologous chromosomes was recognized by scoring for exceptional progeny: X chromosome NDJ was represented by females with normal eyes or *y,v,f; B* males, whereas 4th chromosome NDJ was scored as adults displaying the recessive *ci, ey[R]* phenotype. The formula for the calculation of NDJ frequency is $2 \times \text{exceptional progeny} / (2 \times \text{exceptional progeny} + \text{regular progeny})$.

To determine at which meiotic division(s) the X chromosome NDJ occurred in *myt1* mutant females, *FM7, y, B/y; myt1¹/myt1²* females were crossed to *y+* males (cross 2). NDJ at meiosis I would produce *FM7,y, B/y* or nullo-X eggs, whereas NDJ at meiosis II would result in either *FM7,y,B/FM7,y,B, y/y* or nullo-X eggs. X-chromosome NDJ events resulting in viable progeny were scored as adult females with Bar eyes and yellow bodies (from *FM7,y, B/y* eggs), or double-Bar eye (two copies of the *B* allele), yellow females (from *FM7,y,B/FM7,y,B* eggs) or females with normal eyes and yellow bodies (from *y/y* eggs). The first class is meiosis I NDJ-specific, whereas the latter two classes are meiosis II NDJ-specific progeny.

Results

Molecular and genetic characterization of *Drosophila* Myt1 mutants

Drosophila myt1 is predicted to encode a 61 kDa protein (533 amino acids). Within the kinase domain, Myt1 shares 49% and 47% amino acid sequence identity with *X. laevis* and *H. sapiens* Myt1 homologs (Fig. 1A, purple bar) and 31% sequence identity with Wee1 in the same region (Campbell et al., 1995). Myt1 also shares two conserved domains with other

Myt1 kinases, which are not present in nuclear Wee1 kinases (Lamitina and L'Hernault, 2002; Liu et al., 1997; Wells et al., 1999): a potential trans-membrane domain (Fig. 1A, green bar) and a C-terminal putative Cyclin B interaction motif (Fig. 1A, brown bar).

We isolated two *myt1* mutant alleles (originally designated as *myt1*¹ and *myt1*²) in a genetic screen for hemizygous mutants with phenotypic defects that could be rescued by a *P{myt1}* transgene (Fig. 1B, Materials and methods). These alleles exhibited markedly different viability as hemizygotes; however, these differences were removed by out-crossing, indicating they were due to secondary lesions. Viable hemizygous *myt1* mutants [*myt1*/Df(3L)64D-F] exhibit bristle defects affecting the dorsal thorax, head and eye (not shown), and are male sterile. Although *myt1* females are fertile, we observed variable maternal effect lethal embryonic phenotypes in their progeny (data not shown). Genomic sequencing of the *myt1* alleles identified identical mutations in each: a single nucleotide deletion at position 514 (amino acid 173). The fact that EMS mutagenesis usually causes CG→TA transitions, combined with the unlikelihood that this mutation would have occurred twice independently, suggests that a spontaneous mutation occurred in the previously isogenized stock we used for our screen. The *myt1* mutation is predicted to cause a frame-shift alteration in the sequence of the protein, followed by a premature stop codon at nucleotide 689 (amino acid 232). This would truncate the protein within the kinase domain and also delete other conserved sequence motifs near the C terminus of the protein (Fig. 1C), suggesting that the mutants are likely functionally null. Moreover, *myt1*/Df(3L)64D-F hemizygotes display identical phenotypes as trans-heterozygous combinations of the original alleles, fulfilling classical genetic criteria that these *myt1* alleles are functionally amorphic (Muller, 1932).

Loss of Myt1 causes mitotic proliferation defects during spermatogenesis

The male sterility of *myt1* mutants led us to look for specific cell cycle defects during spermatogenesis. Male germline development begins with stem cell divisions that generate gonialblasts, which then undergo four synchronous mitotic divisions to produce cysts of 16 primary spermatocytes (Fuller, 1993). These primary spermatocytes remain in G2 phase for

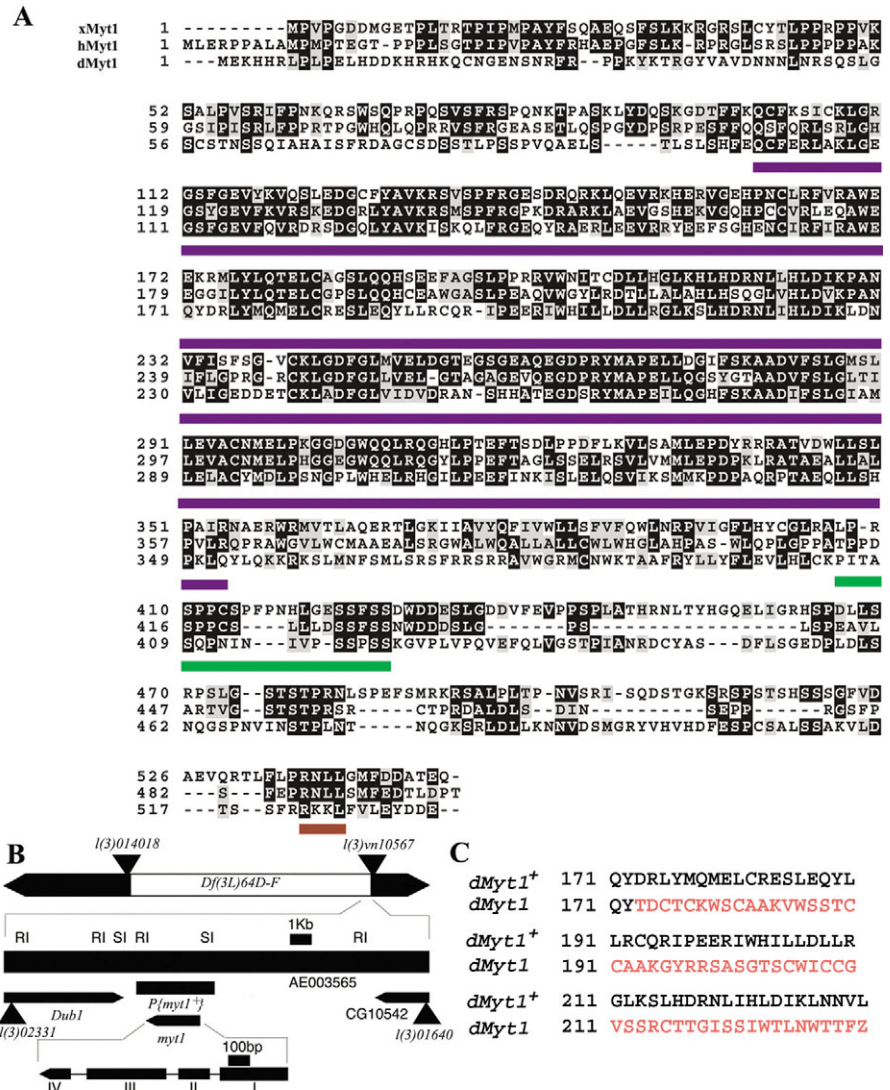
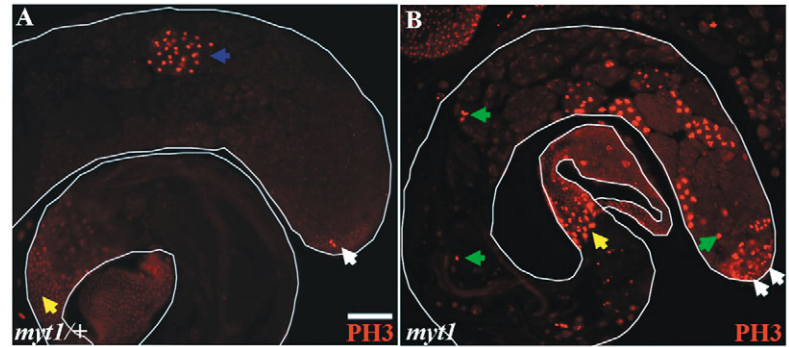


Fig. 1. Genomic and molecular analysis of *myt1*. (A) An alignment of Myt1 sequences from *Xenopus laevis*, *Homo sapiens* and *Drosophila melanogaster*. Black shading indicates identical amino acids and gray shading highlights similar amino acids. The Myt1 protein sequence contains a kinase domain (purple bar), a potential trans-membrane domain (green bar) and a predicted C-terminal Cyclin B interaction motif (brown bar). (B) Physical and genetic map of *myt1* and flanking genes uncovered by Df(3L)64D-F (white box). Black triangles indicate the position of the nearest lethal P-element insertions that complement Df(3L)64D-F. The black bar showing restriction enzyme sites (RI, EcoRI; SI, Sall) represents a 24 kb DNA sequence (coordinates 240,000 to 264,000) in AE003565, a genomic BAC clone. The narrow black bar represents the DNA fragment that was sub-cloned to generate P{myt1}. Black arrow bars represent transcripts of *myt1*⁺ and the nearest flanking genes. Roman numerals indicate exons I to IV of the *myt1* gene. (C) A single base deletion mutation in *myt1*¹ (and *myt1*²) beginning at amino acid position 173 causes a frame-shift, producing an altered amino acid sequence in Myt1 (red letters) that ends with a premature stop codon at position 231.

~90 hours before undergoing meiotic divisions to produce cysts containing 64 syncytial spermatids that differentiate into mature sperm. To analyze how loss of Myt1 function affects these cell divisions, we used an antibody that recognizes a phosphorylated form of histone H3 (PH3) as a marker for mitotic or meiotic cells (Kiger et al., 2000). In control testes, small numbers of mitotic cells were usually seen near the tip

Fig. 2. Cell proliferation defects observed in *myt1* mutant testes. (A) Phospho-histone H3 (PH3, red) antibody staining of *myt1/+* controls, shows mitotic cells at the apical tip (white arrow) and a cyst of meiotic cells (blue arrow) farther along the testis. No additional cells were labeled (yellow arrow). (B) PH3 staining of mitotic cells (white arrows) at the apical tip of the testes in *myt1* mutants as well as additional 16-cell cysts of PH3-positive cells farther along the testes. Isolated mitotic cells (green arrows) were also found along the testis as well as a large cluster of labeled cells at the terminal end (yellow arrow). Scale bar: 20 μ m.



of the testis (Fig. 2A, white arrow). More distally along a control testis, one often observes a single PH3-positive meiotic cyst (Fig. 2A, blue arrow). We observed a striking increase in the numbers of PH3-positive cells in *myt1* mutants (Fig. 2B). In addition to clearly demarcated germline cysts, we also observed isolated PH3-positive cells along the length of *myt1* mutant testes (Fig. 2B, green arrows), as well as PH3-positive cells at the distal end of the testes (yellow arrow) that were never seen in controls (Fig. 2A). These cell proliferation defects were suppressed and male fertility was restored when a *P{myt1⁺}* transgene was introduced into the *myt1* mutant background, confirming that these mutant phenotypes were due to a loss of Myt1 activity. The adult bristle phenotype observed in *myt1* mutants was also rescued by this transgene (not shown).

To determine if there were other proliferation defects observable in the *myt1* mutants, we used BrdU incorporation to assay for DNA replication in short-term (30 minute) cultures of dissected testes. BrdU incorporation was seen only in the cells near the tip of the testes in the controls, implying that pre-meiotic S phase was essentially complete by the time the primary spermatocyte cysts move away from the tip (Fig. 3A). We observed a marked increase in BrdU-incorporating cells near the apical tip of the testes in the *myt1* mutants, relative to controls (Fig. 3A,B). These observations could be explained if cells in the *myt1* mutants cycle faster than normal, or if they continue to cycle instead of undergoing developmental cell cycle arrest. Delays

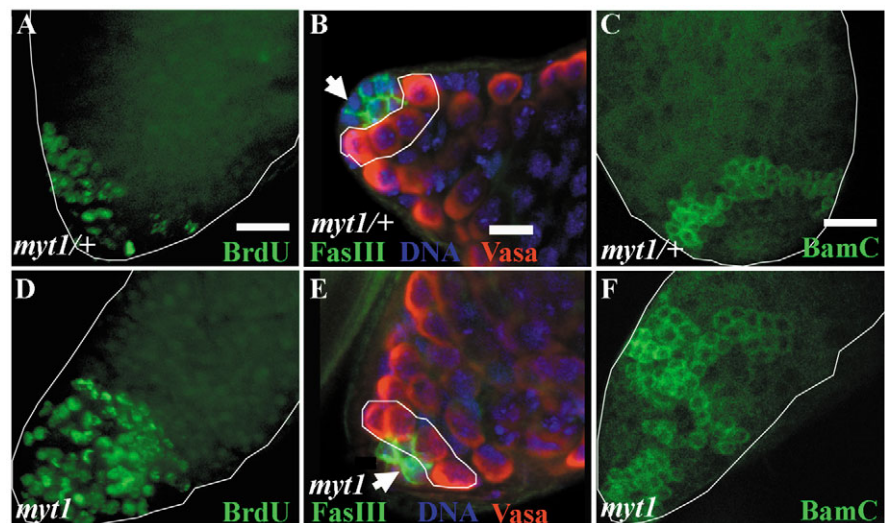
during S phase and mitosis could also contribute to these effects.

Loss of *Myt1* activity affects a developmental switch from mitotic to meiotic cell cycles

Male-sterile mutants with overproliferation defects can result from germline stem cells (GSC) or spermatogonia failing to differentiate properly, so that they continue to proliferate instead of entering meiosis (Gonczy et al., 1997; Kiger et al., 2000; Matunis et al., 1997; Tulina and Matunis, 2001). To determine if overproliferation in *myt1* mutants was attributable to similar defects, we used established cell fate markers to examine germline stem cells, spermatogonia and spermatocytes. *myt1* mutants have normal numbers of germline stem cells, assessed by immunostaining for the germline-specific marker Vasa (Fig. 3B,E) and Fas3, which marks the somatic hub that GSCs associate with (Gonczy and DiNardo, 1996; Lasko and Ashburner, 1990; Patel et al., 1987). There was, however, a significant increase in secondary spermatogonial cells marked by antibodies against BamC in the *myt1* mutants (Fig. 3C,F), relative to controls (Gonczy et al., 1997; Ohlstein and McKearin, 1997). This could occur if the secondary spermatogonia undergo one or more extra rounds of cell division, in which case we would expect PH3-positive spermatogonial cysts with 16 or more cells.

To test this possibility, we performed a BamC and PH3 colocalization experiment. In controls (Fig. 4A), we never observed cysts containing more than eight cells that were both

Fig. 3. Increased numbers of *myt1* mutant secondary spermatogonia. (A,D) BrdU-incorporation labels S-phase cells (green), showing that more cells are labeled in *myt1* mutant testes (D) than in controls (A). (B,E) Fas3 labeling of hub cells (green, arrow) and Vasa germline-specific staining indicates the position of germline stem cells (outlined), showing that there are similar numbers of stem cells in *myt1* mutants (E) and controls (B). (C,F) BamC (green) staining of secondary spermatogonia shows more spermatogonia in *myt1* mutants (F) than in controls (C). Scale bars: 16 μ m for A,C,D,F; 8 μ m for B,E.



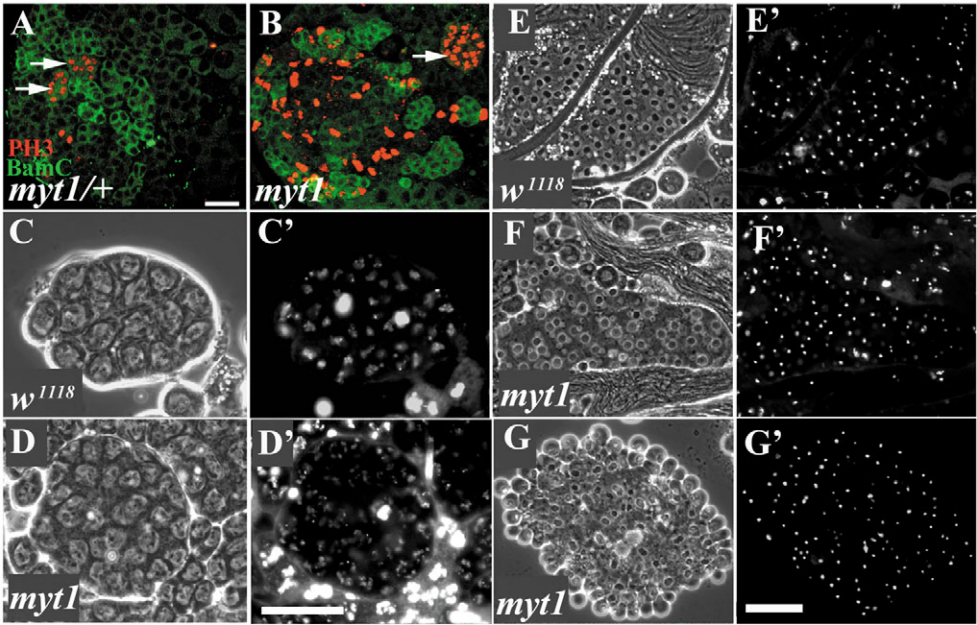


Fig. 4. Secondary spermatogonia can undergo an extra round of mitosis in *myt1* mutants. (A,B) Testes stained with anti-BamC (green) and anti-PH3 (red) antibodies. In the controls, cysts of dividing secondary spermatogonia do not contain more than eight cells (A, white arrows), whereas the *myt1* mutant also contains a 16-cell cyst (B, white arrow), indicating an extra cell division. There were also many scattered PH3-positive cells not co-stained with BamC but intermingled with BamC-positive cells, in the *myt1* mutants. These were probably ectopically dividing somatic cells (see Fig. 5, for explanation). (C–G) Phase-contrast and (C'–G') fluorescence (Hoechst 33258) images of cysts of: (C,C') control 16-cell primary spermatocytes, (D,D') unusual 32-cell spermatocyte cysts seen in *myt1* mutants, (E,E') control 64-cell spermatid cysts, and aberrant-looking 64-cell (F,F') and 128-cell (G,G') spermatid cysts from *myt1* mutants. Scale bars: in A, 20 μ m for A,B; in D', 50 μ m for C,D'; in G', 50 μ m for E–G'.

BamC and PH3 positive, consistent with spermatogonia only undergoing four mitotic divisions. By contrast, ~30% of the *myt1* mutant testes examined ($n=20$) contained at least one 16-cell cyst that was both BamC and PH3-positive (arrow, Fig. 4B), implying that these spermatogonia were undergoing an extra round of cell division. To further test this idea, we examined germ cell cysts by phase contrast microscopy and quantified the numbers of cells in each cyst. As expected, ~10% of the mutant cysts contain twice the expected numbers of primary spermatocytes or spermatids, a phenotype that was never seen in controls (Table 1). Examples of a *myt1* mutant 32-cell cyst of primary spermatocytes (Fig. 4D,D'), 64-cell spermatid cysts (Fig. 4F,F') and 128-cell spermatid cysts (Fig. 4G,G') are shown, along with corresponding 16 cell and 64 cell controls (Fig. 4C,E). Moreover, in both 64-cell and 128-cell *myt1* mutant spermatid cysts, there was consistent evidence of variable, aberrant-looking nuclei and nebenkern (Fig. 4F,G), which were never seen in controls (Fig. 4E). These observations suggest that loss of Myt1 activity affects segregation of chromosomes and mitochondria during meiosis, in addition to the mitotic defects described earlier.

Ectopic divisions in Myt1 mutant germline-associated somatic cells

We next examined whether additional defects in the cell cycle behavior of somatic cells might contribute to the *myt1* overproliferation phenotype. Somatic stem cells located at the apical tip of the testes divide to generate cyst cells whose fate is intimately coupled with male germline development (Gonczy and DiNardo, 1996; Matunis et al., 1997). Two cyst

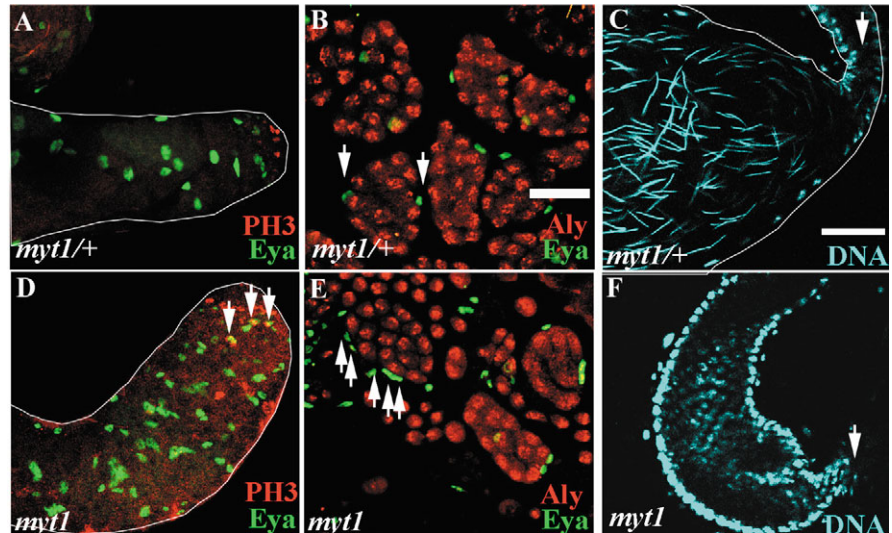
Table 1. Quantification of male germ cell cysts with extra numbers of spermatocytes or spermatids in *myt1* mutant testes

	Number of spermatocytes per cyst		Total
	16 cells/cyst	32 cells/cyst	
<i>w¹¹¹⁸</i>	100	0	100
<i>myt1/Df</i>	36	4	40

	Number of spermatids per cyst		Total
	64 cells/cyst	128 cells/cyst	
<i>w¹¹¹⁸</i>	17	0	17
<i>myt1/Df</i>	28	2	30

cells associate with each gonialblast and remain associated with the descendant cyst for the remainder of spermatogenesis. Normally, these somatic cyst cells do not undergo further cell division, suggesting that their differentiation is coupled with exit from the cell cycle. We used antibodies against Eya to mark the cyst cells (Bonini et al., 1993; Fabrizio et al., 2003). There was a marked increase in the number of cyst cell nuclei in the *myt1* mutants, relative to controls (Fig. 5A,D). Because the cyst cells are quiescent, they are PH3-negative in the controls (Fig. 5A). In *myt1* mutants, however, cyst cell nuclei can be double-labeled with antibodies to PH3 and Eya (Fig. 5D, white arrows). When we used Aly as a marker for spermatocytes (White-Cooper et al., 2000), Aly-positive cysts with more than two Eya-positive cyst nuclei were never observed in controls (Fig. 5B), but were often seen in the mutants (Fig. 5E, arrows), implying that these extra nuclei remain associated with their germline cysts during meiosis. In

Fig. 5. Germline-associated somatic cell nuclei divide ectopically in *myt1* mutant testes. (A,D) Somatic cyst cells stained with anti-Eya (green) and anti-PH3 (B, F, red) antibodies. Increased numbers of cyst cell nuclei were seen in the *myt1* mutants (D, green) relative to controls (A); PH3-positive cyst cells were also detected in the mutants (D, arrows). (B,E) Cyst cells and spermatocytes stained with anti-Eya (green) and anti-Aly (red) antibodies. Extra cyst cells associated with Aly-positive spermatocytes were seen in *myt1* mutants (E, arrows), when compared with controls (B). (C,F) Optical sections through seminal vesicles stained to visualize DNA (blue, arrow shows proximal end of vesicle). No sperm were present in the *myt1* mutant seminal vesicle (F), in contrast to controls, which were full of mature sperm (C). Scale bar: in B, 40 μ m for A,B,D,E; in C, 20 μ m for C,F.



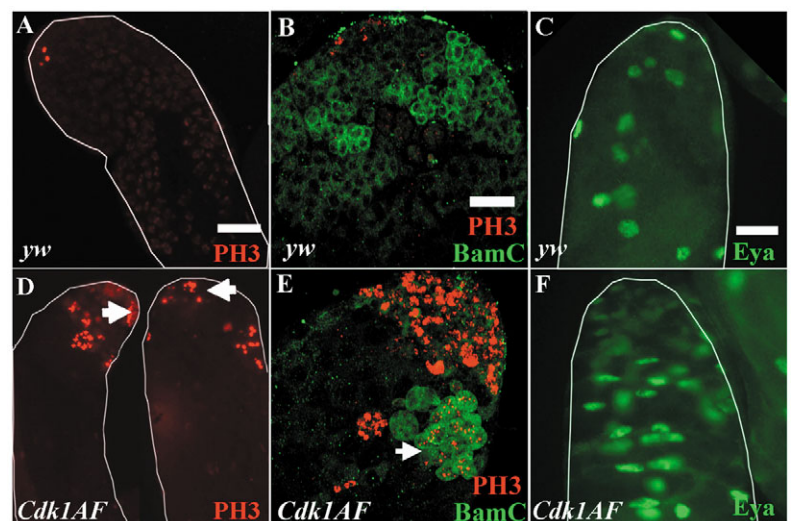
addition to the ectopic division of cyst cells, we also observed that terminal epithelial cells located at the distal end of the testes ectopically label with PH3 antibodies, unlike controls (Fig. 2A,B, yellow arrow). These observations further distinguish *myt1* mutants from previously described male-sterile over-proliferation mutants and implicate Myt1 in a molecular mechanism that promotes cell cycle exit during terminal differentiation. These defects may affect the ability of cyst and terminal cells to provide essential cell signaling or other support functions to their associated germline cells. Such effects could conceivably compromise sperm maturation or translocation and contribute to the observed sterility of male *myt1* mutants. Mature sperm translocate into the seminal vesicle after spermatid differentiation and can be visualized by DNA staining (Fig. 5C). In *myt1* mutants, the seminal vesicle appeared to be empty (Fig. 5F).

A failure of Cdk1 inhibitory phosphorylation is responsible for the overproliferation defects in *Myt1* mutants

Previous studies in *Drosophila* have demonstrated that regulation of entry into mitosis and meiosis is controlled by

inhibitory phosphorylation of Cdk1 (Alphey et al., 1992; Courtot et al., 1992; Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990). Given that Myt1 is a Cdk1 inhibitory kinase, we expected that phenotypic defects of *myt1* mutants would be due to a defect in Cdk1 regulation (Mueller et al., 1995). To test this idea, we expressed a heat shock-inducible, non-inhibitable allele of Cdk1 (*hs-Cdk1AF*) in testes, to see if it would phenocopy any of the defects observed in *myt1* mutants. Expression of this transgene has previously been used to bypass a developmentally regulated G2 arrest in embryonic germline cells (Su et al., 1998). As predicted, heat-shock induced expression of *Cdk1AF* caused germline over-proliferation defects similar to those seen in *myt1* mutants (Fig. 2B). These included increased numbers of PH3-positive cells (Fig. 6D,E), relative to controls (Fig. 6A,B). We confirmed, by BamC antibody co-localization, that some of these PH3-positive cells in the *Cdk1AF*-expressing testes were secondary spermatogonia undergoing an extra round of mitosis (Fig. 6E, arrow). Induction of *hs-Cdk1AF* also phenocopied the defects seen in germline-associated somatic cyst cells labeled with Eya (compare Fig. 6C,F). These results demonstrate that Myt1 inhibitory phosphorylation of Cdk1 is

Fig. 6. The *myt1* mutant over-proliferation defects are due to a failure of Cdk1 inhibitory phosphorylation. (A,D) PH3 (red) staining shows that heat-shocked *Cdk1AF* testes (D) have extra dividing cells, compared with controls (A). (B,E) BamC (green) and PH3 (red) staining identifies dividing secondary spermatogonia. In the heat-shocked *Cdk1AF* testes, a 16-cell cyst undergoing ectopic mitosis (E, white arrow) is shown, not seen in the controls (B). (C,F) Eya (green) staining to label somatic cyst cells shows that heat-shocked *Cdk1AF* testes have more cyst cells (F) than do controls (C). Scale bar: 40 μ m for A,D; 8 μ m for B,E; 20 μ m for C,F.



required for regulating multiple aspects of cell cycle behavior during spermatogenesis.

Loss of dMyt1 activity also results in germline cell cycle defects during oogenesis

Female *myt1* mutants were fertile; however, a high incidence of early lethality in maternally affected mutant embryos (not shown) suggested that Myt1 might also function during oogenesis. Oogenesis initiates with stem cell divisions that produce cystoblasts that then undergo four synchronous mitotic divisions, to generate 16 cell cysts (Spradling, 1993). A single cell in each cyst differentiates into an oocyte and progresses into prophase of meiosis I, where it remains arrested until ovulation. The 15 remaining cells in the cyst differentiate as nurse cells. Two or three germline stem cells (GSCs) are located at the tip of the germarium, each containing a ball-shaped fusome-related structure called a spectrosome (Lin and Spradling, 1995). Fusomes are germline-specific membranous organelles that interconnect the cyst cells and are thought to coordinate their mitotic cell divisions. Using the position of GSCs and antibodies against Hts to label spectrosomes (Lin et al., 1994; Zaccari and Lipshitz, 1996), we determined that the number of GSCs was comparable in *myt1* mutants and controls (Fig. 7A,B, inset a1, b1, green arrows). We also counted the numbers of dividing GSCs, cystoblasts and cystocytes and calculated a mitotic index for each cell type (Table 2), using antibodies against Cnn and Hts to mark spindle poles and spectrosomes (or fusomes, in cystocytes), respectively, as well as the DNA-labeling dye Hoechst 33258 to mark condensed mitotic chromosomes (Fig. 7A-D, outlined areas). Female GSCs typically undergo one cell cycle per day, consequently mitotic GSCs are rarely observed and the mitotic index is very low (Lin and Spradling, 1993). We found only two mitotic GSCs in 130 germaria from control ovarioles (Table 2). By contrast, we found 15 mitotic GSCs among 50 *myt1* mutant germaria, a 20-fold increase in the mitotic index. The mutant cystoblasts and their cystocyte descendants also had a significantly higher mitotic index than normal (Table 2), so it was not uncommon to find a metaphase stem cell (green arrows) and a metaphase cystoblast or cystocyte (yellow arrows) in a single *myt1* mutant germarium (Fig. 7D). In the controls, at most a single dividing cyst was seen in each germarium (Fig. 7A,C). These results show that loss of *myt1* activity causes germline overproliferation in females, as well as males. We did not observe egg chambers with greater than 16 cells in *myt1* mutants, however, indicating that ectopic germline cell divisions did not account for this defect, in females.

Oocyte and nurse cell differentiation appeared normal in the *myt1* mutants, as assessed by antibody staining of Orb and Gurken in oocytes (Schupbach and Roth, 1994), and by nuclear morphology of the nurse cells (not shown). When *myt1* mutant

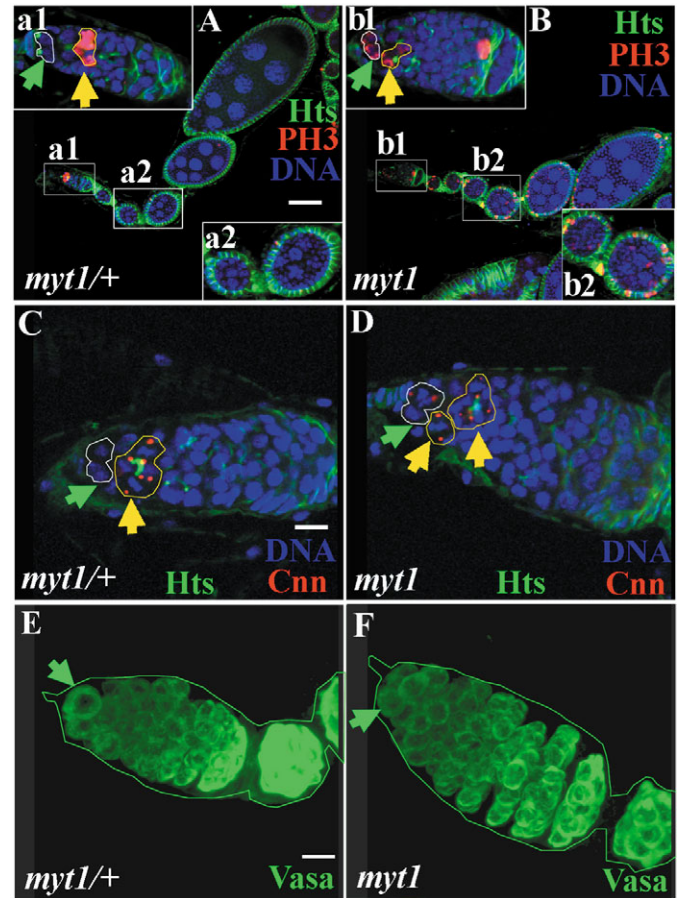


Fig. 7. Mitotic over-proliferation defects seen in *myt1* mutant ovaries. (A,B) Ovarioles labeled by antibodies against PH3 (red) to mark mitotic cells, Hts (green) to mark spectrosomes and stained with Hoechst 33258, marking DNA (blue). In *myt1* mutants (B), germaria often contained both dividing stem cells (green arrow) and dividing cystocytes (yellow arrow), not seen in controls (compare insets: a1, b1). The *myt1* mutants also had more PH3-positive somatic follicle cells surrounding the egg chambers than did controls (compare insets: a2, b2). (C,D) Centrosomin staining (Cnn, red) for centrosomes, Hts (green) staining for fusomes and DNA (blue) staining showed significantly more metaphase stem cells (green arrow) and cystocytes (yellow arrows) in *myt1* mutants (D) compared with controls (C). (E,F) Stacked confocal images of a Vasa-stained germarium, showing that *myt1* mutant germaria (F) contain more cysts than controls (E). The *myt1* mutant germline stem cells (F, green arrows) were also slightly smaller than controls (E). Scale bar: 40 μ m for A,B; 8 μ m for C-F.

germaria were examined with antibodies against the Vasa germline marker, we noted a significant increase in the number of cysts (one- to twofold, $n=20$), relative to controls (Fig.

Table 2. Quantification of the mitotic index associated with different cell types in *myt1* mutant ovaries

Genotype	Mitotic index				
	Stem cell	Cystoblast	Two-cell cystocyte	Four-cell cystocyte	Eight-cell cystocyte
<i>myt1</i> /+	0.015 ($n=130$)	0.000 ($n=62$)	0.032 ($n=62$)	0.048 ($n=62$)	0.016 ($n=62$)
<i>myt1</i>	0.300 ($n=50$)	0.160 ($n=50$)	0.160 ($n=50$)	0.160 ($n=50$)	0.180 ($n=50$)

The mitotic index was calculated as the number of PH3-positive cells divided by the total number of cells, for each indicated cell type (scored by criteria that are illustrated in Fig. 6). The number of germaria counted for each sample is indicated in brackets.

Table 3. Non-disjunction at meiosis I in *myt1* mutant females

Female genotypes	Regular progeny	Regular progeny	Diplo-X progeny	Null-X progeny	% of X-NDJ	4 ⁴	% of 4-NDJ	Total progeny
Cross 1	X/O	XX [^] Y	XX	X [^] Y				
<i>myt1</i> ¹ / <i>myt1</i> ²	135	144	5	13	11.40	1	0.70	315
<i>myt1</i> ¹ / <i>TM3</i>	459	340	0	1	0.10	0	0	801
Cross 2	y ⁺ females	y males	<i>FM7</i> ,y,B/ y females	y ⁺ males	% of X-NDJ	<i>FM7</i> ,y,B/ <i>FM7</i> , y,B or y/y females		
<i>FM7</i> ,y,B/y; <i>myt1</i> ¹ / <i>myt1</i> ²	300	279	51	63	28.25	0		807
<i>FM7</i> ,y,B/y; <i>myt1</i> ¹ / <i>TM3</i>	347	360	1	4	1.39	0		717

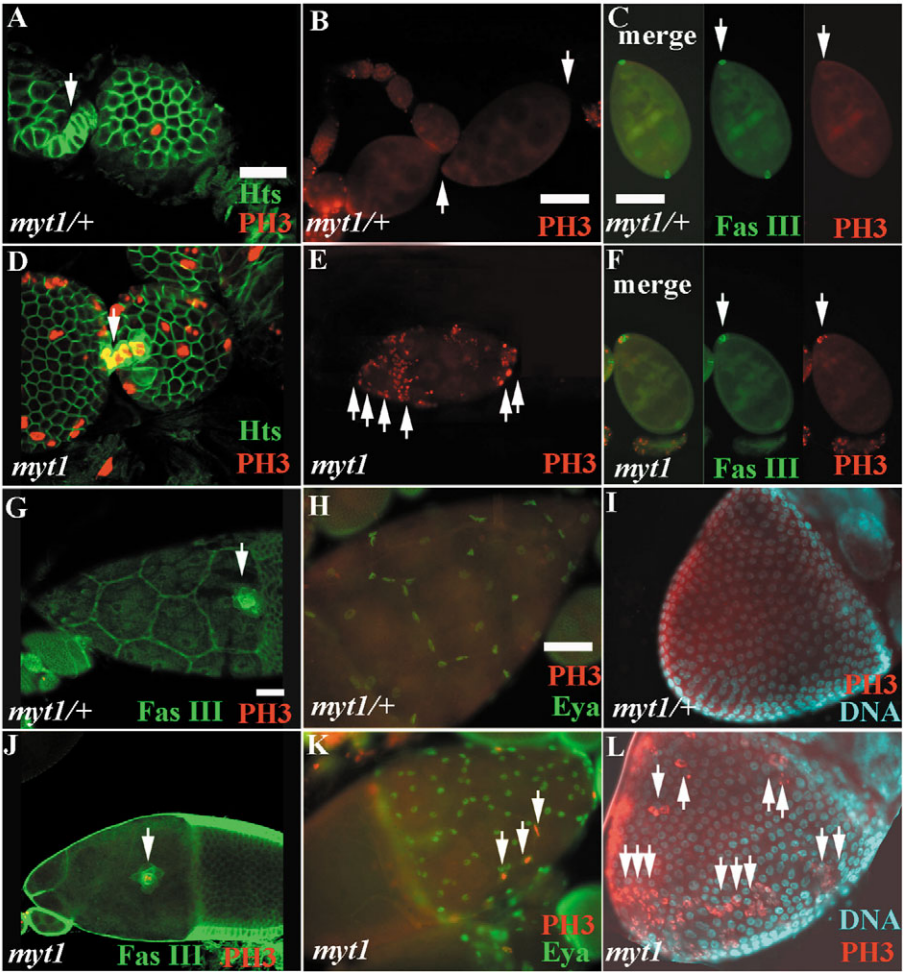
The genetic crosses and calculations used to generate the data in this table are described in the Materials and methods. The percentage of NDJ is higher in cross 2 than in cross 1 because of the introduction of the *FM7* balancer chromosome, which by itself increases the frequency of NDJ in both controls and in *myt1* mutants.

7E,F). Although *myt1* mutant cystoblasts and cystocytes were similar in size to controls, the GSCs appeared slightly smaller (compare Fig. 7E with F, green arrows). These data suggest that *myt1* mutant GSCs were cycling more rapidly and therefore produced more germline cysts. As the differences were not as extreme as the GSC mitotic index measurements would predict, these results also imply that compensatory delays probably occur during these mitotic cell cycles that can account for this discrepancy.

To investigate whether there were effects on homologous chromosome segregation during female meiosis in *myt1* mutant

females, standard genetic tests were undertaken to identify non-disjunction (NDJ) events, as described in the Materials and methods (Giunta et al., 2002). These tests showed that loss of Myt1 causes elevated non-disjunction, implicating Myt1 as a regulator of female meiosis. The NDJ frequency for *myt1* mutants was much higher than controls for the X chromosome and the 4th chromosome (Table 3, cross 1). Further experiments indicated that exceptional progeny derived from NDJ during meiosis I in *myt1* females (Table 3, cross 2). These data demonstrate that loss of Myt1 activity compromises female meiosis, specifically meiosis I.

Fig. 8. Follicle cell defects observed in *myt1* mutant ovarioles at different stages of development. (A,D) Stage 2 egg chambers stained for Hts (green) and PH3 (red). Stalk cells (arrow) are PH3-positive in ~90% of *myt1* mutants at this stage (D), but are PH3-negative in controls (A). (B,E) Stage 7 egg chambers stained for PH3 (red). Follicle cells at each end of the egg chamber (arrows) are PH3 positive in *myt1* mutants (E) but PH3 negative in controls (B). (C,F) Stage 7 egg chambers stained for Fas3 (green) and PH3 (red). Arrow indicates a PH3-positive polar cell (seen in 100% of *myt1* mutants, F), not seen in controls (C). (G,J) Stage 10A egg chambers stained for Fas3 (green) and PH3 (red). Arrow in J indicates a PH3-positive border cell (seen in ~20% of *myt1* mutants), not present in controls (G). (H,K) Stage 10A egg chambers stained for Eya (green) and PH3 (red). Arrows indicate PH3-positive, Eya-positive stretched cells in ~16% of *myt1* mutants (K) not seen in controls (H). (I,L) Stage 10B egg chambers stained for DNA (cyan) and PH3 (red). Arrows indicate PH3-positive main body follicle cells in ~6% of *myt1* mutants (L) but not in controls (I). Scale bar: 8 μ m for A,D; 40 μ m for B,C,E-G,J; 20 μ m for H-L.



Germline-associated follicle cells also undergo ectopic cell division in *Myt1* mutants

Female germline cells are associated with somatic follicle cells derived from stem cell precursors (Margolis and Spradling, 1995). When each 16-cell germline cyst buds off from the germarium as an egg chamber, a layer of undifferentiated follicle cells surrounds it. These follicle cells then differentiate into functionally distinct subclasses. The stalk cells (located between each egg chamber) and the polar cells (located at each end of the chamber), cease dividing immediately after the egg chamber forms, whereas the remaining follicle cells proliferate asynchronously until stage 6 of oogenesis (Bai and Montell, 2002). Accordingly, early egg chambers have only small numbers of PH3-positive follicle cells (inset a2, Fig. 7A; Fig. 8A, red). In *myt1* mutant egg chambers, there was a marked increase in PH3-positive follicle cells before stage 6 (Fig. 7C, inset c2; Fig. 8D), as well as ectopic PH3-positive follicle cells after stage 6 (compare Fig. 8B with 8E, arrows). Curiously, these ectopic PH3-positive follicle cells primarily appeared at the anterior and posterior ends of each egg chamber (Fig. 8E).

Each of the four major types of follicle cells can be distinguished by their cell shape and by expression of distinct molecular markers. Stalk cells have a unique disc-like shape and inter-egg chamber location (Fig. 8A, arrow); polar cells are located at the end of each egg chamber and express Fas3 before stage 9 (Fig. 8C, green); border cells maintain Fas3 expression and migrate towards the posterior after stage 9 (Fig. 8G); and stretched cells extend over the 15 nurse cells and express Eya (Fig. 8H). By these criteria, the different follicle cell types all appeared to be represented in *myt1* mutants; however, unlike the controls, some of these cells were PH3 positive, suggesting that they were undergoing ectopic cell divisions (Fig. 8D-F, arrows Fig. 8J,K). Consistent with this interpretation, there were more Eya-expressing cells in the mutants than in controls by stage 9 (compare Fig. 8H,K), indicating that some of these cells were able to complete cell division. The typical 'stretched' morphology characteristic of this cell type was disrupted, presumably as a result of cytoskeleton reorganization accompanying mitosis (Fig. 8H,K, arrows). We also observed ectopic PH3-positive main body follicle cells in mutant egg chambers after stage 9, long after these cells normally cease dividing (Fig. 8I,L). Thus, loss of Myt1 function causes germline-associated somatic cells to undergo ectopic cell division, in both males and females.

Discussion

Regulation of Cdk1 by inhibitory phosphorylation is critical for cell survival and for the developmental regulation of cell proliferation. Previously, we have demonstrated that *Drosophila* Wee1 serves an essential role in a pre-mitotic checkpoint that operates during the rapid S/M nuclear divisions of early embryogenesis (Price et al., 2000; Stumpff et al., 2004). The cell cycle timing defects observed in these mutants are consistent with the proposal that Wee1 serves a conserved role in protecting nuclei from cytoplasmically activated Cdk1 (Heald et al., 1993). In the present report, we demonstrate that Myt1 serves distinct cell cycle regulatory functions, required for the fidelity of specific stages of development.

Overexpression studies in several systems suggest that Myt1 can influence the timing of G2/M transitions (Cornwell et al.,

2002; Price et al., 2002; Wells et al., 1999). Loss-of-function studies now demonstrate that Myt1 serves distinct Cdk1 regulatory functions that are essential for normal gametogenesis and for adult bristle development (not shown). Consistent with previous studies in other organisms that implicated Myt1 in female meiosis (Kalous et al., 2005; Karaïskou et al., 2004; Okano-Uchida et al., 2003; Okumura et al., 2002; Palmer et al., 1998; Peter et al., 2002), we observed a marked elevation of meiotic chromosome segregation defects in the progeny of female *myt1* mutants. Female *myt1* mutants are fertile in spite of these segregation defects; however, many of their progeny undergo variable embryonic lethality (not shown). In male *myt1* mutants, loss of Myt1 function results in complete sterility. This phenotype appears to be due to defects during both meiosis and spermatid differentiation in male *myt1* mutants. Normally, *Drosophila* spermatocytes undergo a prolonged G2 phase arrest, which allows time to synthesize cellular components required for subsequent development, before the onset of meiosis I cell division. Oocytes do not undergo a similar growth phase, owing to specialization of the germline nurse cells, which synthesize the mRNAs and proteins required for egg development. Thus, unlike spermatocytes, *Drosophila* oocytes almost immediately progress into prophase of meiosis I after completing the four mitotic divisions that produce the 16-cell cyst (Spradling, 1993). These differences in male and female germline development may explain the differences in requirements for Myt1 activity that we observe. Moreover, our data suggest that Myt1 has evolved specific functions that are important for developmentally regulated growth phases. This hypothesis is also consistent with the requirement for Myt1 in *Xenopus* and *A. pectinifera* during the prolonged 'G2-like' prophase arrest of early oocytes, which also involves extensive cell growth and synthesis of proteins and mRNAs required for subsequent embryonic development (Furuno et al., 2003; Karaïskou et al., 2004; Nakajo et al., 2000; Okano-Uchida et al., 2003; Okumura et al., 2002; Palmer et al., 1998; Peter et al., 2002).

In addition to confirming that Myt1 serves a conserved role in regulating meiosis, our studies of *myt1* mutants also provide evidence that Myt1 serves novel functions that affect mitotic cell proliferation during both male and female gametogenesis. Mitotic index measurements of female germline cells suggest that loss of Myt1 activity influences the timing of the mitotic cell cycles that precede meiosis, although live analysis will be needed to verify this conclusion. Delays in mitosis may also contribute to this phenotype, as the observed increase in numbers of germline cysts in *myt1* mutant germaria was not directly proportional to the increased mitotic index seen in germline stem cells.

A further unexpected mitotic defect associated with loss of Myt1 activity involved ectopic divisions of germline-associated somatic cells, seen in both males and females. As germline-associated somatic cells normally become quiescent as they differentiate, this observation suggests a role for Myt1 in a molecular mechanism that allows or facilitates exit from the cell cycle. An additional mitotic defect was identified in male *myt1* mutants, which was not seen in females. Approximately 10% of the cysts undergo an extra round of mitotic cell division before cells differentiate into primary spermatocytes, suggesting that Myt1 also affects the fidelity or timing of this developmentally regulated cell fate decision.

However, unlike previously described male-sterile overproliferation mutants with cell fate defects (Gonczy et al., 1997; Kiger et al., 2000; Matunis et al., 1997; Tulina and Matunis, 2001), the majority of the *myt1* mutant cysts do not undergo such ectopic mitotic divisions. Collectively, these data suggest that Myt1 serves distinct Cdk1 regulatory functions that coordinate cell cycle behavior with important developmental transitions. Precisely how Myt1 accomplishes these diverse functions is unknown; however, our results are consistent with the idea that Myt1 specifically regulates Cdk1 activity in the cytoplasm (Liu et al., 1997; Wells et al., 1999).

Strikingly, RNAi depletion of Myt1 in cultured *Drosophila* cells was previously reported to cause a marked disruption of the Golgi apparatus (Cornwell et al., 2002). This observation suggests an intriguing possibility. As the Golgi apparatus serves a key role in trafficking and secretion of proteins in rapidly growing cells, perhaps Myt1 regulation of Cdk1 activity might indirectly affect the biosynthesis and assembly of subcellular structures required for crucial developmental transitions affected in *myt1* mutants.

According to current models describing regulation of the G2/M transition, Myt1 and Wee1 inhibit Cdk1 in the cytoplasmic and nuclear compartments during interphase, respectively, ensuring a complete block to mitotic progression. Once cells are ready to divide the regulatory proteins that trigger mitosis are thought to first activate Cdk1 at the centrosomes, initiating a self-amplifying wave of Cdk1 activation that sweeps through the cytoplasm and into the nucleus (Jackman et al., 2003; Kramer et al., 2004). This mechanism ensures that early mitotic events are coordinated throughout the cell. Loss of Myt1 activity would be expected to disrupt this coordination by allowing premature activation of Cdk1 in the cytoplasmic compartment, even if Wee1 can still protect the nucleus from active Cdk1. We are now in an excellent position to test key assumptions of this model in vivo, using *wee1* and *myt1* mutants to determine how loss of these Cdk1 inhibitory kinases affects specific cell structures and organelles, at different stages of the cell cycle.

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