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Drosophila Embryonic Cell-Cycle Mutants

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ABSTRACT Nearly all cell division mutants in Drosophila were recovered in late larval/pupal lethal screens, with less than 10 embryonic lethal mutants identified, because larval development occurs without a requirement for cell division. Only cells in the nervous system and the imaginal cells that generate the adult body divide during larval stages, with larval tissues growing by increasing ploidy rather than cell number. Thus, most mutants perturbing mitosis or the cell cycle do not manifest a phenotype until the adult body differentiates in late larval and pupal stages. To identify cell-cycle components whose maternal pools are depleted in embryogenesis or that have specific functions in embryogenesis, we screened for mutants defective in cell division during embryogenesis. Five new alleles of Cyclin E were recovered, ranging from a missense mutation that is viable to stop codons causing embryonic lethality. These permitted us to investigate the requirements for Cyclin E function in neuroblast cell fate determination, a role previously shown for a null Cyclin E allele. The mutations causing truncation of the protein affect cell fate of the NB6-4 neuroblast, whereas the weak missense mutation has no effect. We identified mutations in the pavarotti (pav) and tumbleweed (turn) genes needed for cytokinesis by a phenotype of large and multinucleate cells in the embryonic epidermis and nervous system. Other mutations affecting the centromere protein CAL1 and the kinetochore protein Spc105R caused mitotic defects in the nervous system.

KEYWORDS Drosophila melanogaster cell cycle pav tum cyclin E cal1 spc105R

As organisms progress from a single cell, fertilized embryo to a multicellular adult, cell division must be coordinated with developmental cues. Identification of mutants defective in cell division provides an entry point for elucidating the regulatory signals between division and differentiation. Such mutants also can reveal unique cell-cycle control used to achieve particular developmental strategies.

Drosophila development requires that two body plans be constructed: a larval body and an adult body, the latter built from differentiating imaginal cells during pupation as larval tissues are histolyzed. Embryogenesis produces both the larval body and the imaginal cells. In embryogenesis 13 rapid divisions occur in a nuclear syncytium under maternal control (Figure 1). After cellularization and the onset of zygotic gene expression, there are three additional mitotic divisions in most embryonic cells, the postblastoderm divisions. At this developmental transition, the cells of the epidermis exit the cell cycle, cells in larval tissues enter the endocycle (an S-G cycle that increases ploidy), whereas mitotic divisions continue in the nervous system. During larval development, mitotic divisions occur only in the nervous system and in diploid imaginal tissues.

Nearly all Drosophila cell-cycle mutants recovered from forward genetic screens were identified by a phenotype of late larval/pupal lethality due to a failure of cell division in imaginal tissues (Gatti and Baker 1989; Gatti and Goldberg 1991). Maternal stockpiles of essential mitotic proteins were presumed to accommodate the embryonic mitotic divisions in these mutants. Because larval tissues grow by increasing ploidy, and thus cell size, cell division defects in the imaginal tissues are not manifested until the imaginal cells begin to differentiate to form the adult body.

Fewer than 10 cell-cycle mutants with embryonic lethality have been identified. Most of these are the consequence of mutations in genes encoding proteins that are turned over in the cell cycle, thus eliminating maternal pools. Examples of these include the Cyclins A and E, the Cdc20 homolog Fizzy, the Securin Pimples, and the Separa subunits Three Rows (D’Andrea et al. 1993; Knoblich and Lehner 1993; Knoblich et al. 1994; Leismann et al. 2000; Sigrist et al. 1995; Straumann and Lehner 1996). Other mutants have an embryonic phenotype because their protein products are required to alter the cell
cycle or cell division in response to developmental cues, such as the addition of cytokinesis after cycle 13 (Hime and Saint 1992; Lehner 1992), the cessation of mitosis by the Cdh1 homolog Fizzy Related (Sigrist and Lehner 1997), or action of the Cdk inhibitor Dacapo to add a G1 cell cycle phase (de Nooij et al. 1996; Lane et al. 1996). A mutation in a subunit of the Condensin complex causes mitotic defects in the nervous system late in embryogenesis, possibly because the maternal stockpiles become depleted by continued mitosis in the maternal stockpiles. The transition occurs after division cycle 16 (Figure 1), is under developmental control, and is stereotypically patterned (Duronio and O’Farrell 1994; Smith and Orr-Weaver 1991). This transition is driven by transcriptional activation of multiple genes required for S phase, permitting the maternal stockpiles to be used to drive the G1-S transition in embryos from mutant lines (Figure 2) (Duronio and O’Farrell 1994). Because we visually examined the in situ hybridization pattern in individual embryos, we identified embryos in which the postblastoderm divisions were not completed and thus had reduced cell number, embryos with large cells in the CNS or epidermis, as well as embryos with developmental patterning defects (Figure 2, D and E).

Isogenized cn bw sp homozygous males were fed 35 mM ethyl methanesulfonate (EMS) and crossed to females homozygous for a dominant temperature-sensitive mutation (DTS91 pr cn) and a CyO balancer with a lacZ gene under the control of the Ubx promoter. Single balanced isolines were established by selecting against the DTS91 chromosome at 29° as detailed in Royzman et al. (1997). The third chromosome lines analyzed were established in the laboratory of Ruth Lehmann (Skirball Institute) (Moore et al. 1998). These also were EMS mutagenized by the use of an isogenized ru st P[(af-lacZ) e ca chromosome. The third chromosome lines were balanced over TM3 Sh, P[Ubx-lacZ]. The second chromosome lines were estimated to have 2.7 lethal mutations per chromosome and third chromosome lines 1.9.

For screening, 8- to 15-hr embryos were collected from each mutant line, fixed, and hybridized to riboprobes for lacZ and PCNA.

The Ubx-lacZ pattern (Figure 2C) distinguished heterozygous embryos with the balancers from the homozygous mutant embryos. The fag-lacZ reporter is expressed in the germ cells and did not interfere with analysis of the PCNA in situ pattern. The in situ hybridization was performed with multwell baskets to analyze 144 lines at a time, as described (Royzman et al. 1997).

**Phenotypic analysis**

The mutants producing embryos with putative postblastoderm division defects and large cells in the central nervous system (CNS) or epidermis initially were retested by DAPI staining (1 μg/ml) to visualize all embryonic nuclei. Those with apparent cell-cycle defects were characterized further by the incorporation of 5-bromo-2-deoxyuridine to examine S-phase (Smith and Orr-Weaver 1991) and by staining with antibodies to phospho-H3 and tubulin to analyze mitosis (Dej et al. 2004). The thoracic vs. abdominal fate of the NB6-4 neuroblast in the Cyclin E mutants was determined by staining with antibodies for Eagle [rabbit antibody diluted 1:1000 (Higashijima et al. 1996)] and Repo [mouse antibody diluted 1:10 (DSHB)], as described by Akiyama-Oda et al. (2000) with modifications. Embryos were fixed in a mixture of 4% paraformaldehyde in 1X phosphate-buffered saline (PBS) with heptane (at 1:1 ratio) and then devitellinized with 100% methanol. After rehydration into 1X PBSTr (1X PBS plus 0.1% TritonX-100) the samples were blocked for 1 hr in blocking/antibody incubation buffer (1X PBSTr, 2% each of goat and donkey serum). After overnight incubation with primary antibody at 4°, the embryos were washed in 1X PBSTr then bound with antirabbit or antimouse secondary antibodies (1:500; Jackson ImmunoResearch) at room temperature for 1 hr. Stained embryos were mounted in Vectashield and examined with a Zeiss LSM 510 confocal system.

**Mutant mapping**

The mutants were mapped by meiotic recombination with visible markers, male recombination (Chen et al. 1998), and by complementation tests using the Bloomington Chromosome 2 and 3 deficiency kits. Precise mapping of cal122k12 was done with small deletions generated by Parks et al. (2004). In complementation tests we first scored for lethality and then for the embryonic PCNA in situ phenotype (for Cyclin E alleles) or the DAPI embryonic phenotype. The cyclin E32-8 and tum1520-20 mutations were recovered on the same chromosome and were separated by meiotic recombination.

**Molecular characterization**

The mutant alleles were sequenced from single embryo DNA preps (Strumpf and Volk 1998) by squashing the embryo in 25 μl of Gloor

![Figure 1 Schematic of cell cycle changes in Drosophila embryogenesis. There are 13 rapid S-M divisions in the Drosophila embryo under maternal control, followed by three postblastoderm divisions. These latter divisions follow the onset of zygotic transcription, are controlled by zygotic expression of the string cdc25 phosphatase, and are slowed by the presence of a G2 phase in the cell cycle. After these divisions, cells in the epidermis exit the cell cycle while cells in differentiating larval organs add a G1 phase and enter the endocycle. Subsequent G1-S transitions are marked by transcriptional induction of genes required for S phase, the basis for our genetic screen by in situ hybridization of PCNA. Mitotic divisions continue in the developing nervous system. (MBT, Midblastula transition, the transition from maternal to zygotic control).](image-url)
RESULTS AND DISCUSSION

Summary of screen

A total of 3010 EMS-mutagenized second chromosomes and 1000 EMS-mutagenized third chromosomes were screened for alterations in the PCNA in situ pattern in embryos (Park 2006; Royzman et al. 1997). Transcription of the PCNA gene is induced when the first G1 phase occurs in embryogenesis and occurs in a dynamic pattern coincident with the onset of S phase in the endocyte (Figure 1 and Figure 2) (Duronio and O’Farrell 1994).

A total of 201 mutants exhibited aberrant PCNA expression. For approximately 75% of these (148), this was likely a consequence of developmental abnormalities, as embryonic morphology was grossly disrupted. The remaining 53 mutants fell into several categories: (1) 15 reduced levels of PCNA transcripts. Seven of these were shown to be alleles of the E2F transcription factor subunits dDP and dE2F1 that promote PCNA transcription (Royzman et al. 1999; Royzman et al. 1997). (2) A total of 18 had defects in the postblastoderm divisions that were detectable in the PCNA in situ pattern (Figure 2D). Those mutations that were cloned or shown to correspond to known genes have been described in previous publications. We recovered four alleles of dup (Cdt1) (Whittaker et al. 2000), one of comp-C (Heeger et al. 2005), two of the condensin subunit dacp-g (Dej et al. 2004), one of thr (a subunit of Drosophila Separase) (Dej et al. 2004), and two of pim (the Drosophila Securin) (Dej et al. 2004). (3) A total of 10 mutants had large cells in the central nervous system (Figure 2E). (4) A total of 10 mutants had persistent PCNA transcripts in the endocyte domains (Figure 2F). Here we focus on mutants from the last two categories that were molecularly characterized.

New cyclin E alleles

Mutants were recovered whose PCNA transcripts failed to be down-regulated in the anterior and posterior midgut. Five of these mapped to the second chromosome, as does the Cyclin E gene, when mutated causes persistent PCNA transcript in the endocyte domains (Figure 2F) (Duronio and O’Farrell 1995; Sauer et al. 1995). These five were shown to be alleles of Cyclin E by complementation tests with the Cyclin E1f36pim lethal allele, as well as Df(2L)TE35D-1, which deletes the Cyclin E gene. The putative alleles were lethal over both the deficiency and the Cyclin E1f36pim allele. The lethal phase suggested varying strength of the alleles, as cyclin E1f36pim, cyclin E2f32a, and cyclin E1f137 were embryonic lethal over the deficiency, whereas cyclin E1f23 and cyclin E1f29 were lethal over the deficiency. In addition to failure to complement for lethality, we confirmed that the trans-heterozygous embryos exhibited the mutant PCNA in situ phenotype. Moreover, the cyclin E1f29-25a, cyclin E2f3, and cyclin E1f23.8 alleles were found to be viable with rough eyes and female sterile in trans to the Cyclin E2f female-sterile allele.

Four of the five new Cyclin E mutations were sequenced and the predicted protein changes identified (Table 1). The severity of the protein changes matched the phenotypic analysis, as the three strongest alleles are expected to cause truncated proteins (Table 1). The cyclin E1f36 missense allele has been extensively characterized for its effects on follicle cell amplification during ovarian development (Park et al. 2007).

Neuroblast identity requires normal cyclin E function

In embryos homozygous mutant for the null allele of Cyclin E, cyclin E1f36, other workers found that the identity of a specific neuroblast was altered (Berger et al. 2005). In the thorax, NB6-4t divides asymmetrically to produce neurons and glia, whereas in the abdomen the corresponding neuroblast, NB6-4a, divides symmetrically to generate glia progeny. Strikingly, in the Cyclin E allele tested, NB6-4t divides.
symmetrically. This effect was proposed to be independent of the cell-cycle function of Cyclin E, as other cell-cycle regulators did not cause this transformation in neuroblast fate (Berger et al. 2005). We exploited the new alleles of Cyclin E to test whether loss of Cyclin E function consistently caused this change and whether the extent of neuroblast transformation would correlate with allele strength. We examined two of the new Cyclin E alleles that are predicted to cause truncated proteins and observed they also caused NB6-4t to be transformed to NB6-4a (Figure 3). In contrast, no fate transformation occurred in the weak cyclin E1f36 allele (Table 2), indicating this allele retained sufficient Cyclin E function for neuroblast determination. The observation that all strong alleles of Cyclin E tested caused the same defect in neuroblast fate demonstrates that necessity of Cyclin E function, although it does not permit us to distinguish whether this is mediated by its cell-cycle function or an additional activity of Cyclin E.

**New alleles of cell division genes**

We investigated five of the mutants exhibiting cells with large nuclei in the nervous system or epidermis (Table 1). Two of these had cells with large nuclei throughout the CNS and in the epidermis, whereas three had large nuclei in isolated cells in the CNS (Figure 4). Mutations IR8 and 3C157 were found to be in the same complementation group. These five mutants were isogenized and mapped.

Mutant 3C53 was mapped to the interval 63E1-2;64B1 by failure of Df(3L)GN50 to complement the large nuclei phenotype. Because the cytokinesis gene pavarotti (pav) is in this interval, we tested pavB200 and found that this also failed to complement (Somers and Saint 2003). In addition, we observed multinucleate cells in the CNS of 3C53 mutant embryos, consistent with cytokinesis defects. Thus, we conclude that 3C53 is an allele of the pav gene, which encodes a kinesin motor protein.

Mutant 32a-20 also exhibited defects consistent with cytokinesis failure. The mutant was uncovered by Df(2R)CX1 and delineated to a region of 10 genes by male recombination. One of these genes is tumbleweed (tum), encoding RacGAP50C, a Rho family GTPase required for cytokinesis (Zavortink et al. 2005). Indeed, sequencing revealed that the mutation caused a Leu to His substitution in a conserved residue in the GAP catalytic domain box II (Table 1).
The three remaining mutations were found to affect centromere and kinetochore proteins (Table 1). Mutant 2k32 was mapped to the region 89E11-F1 by its failure to complement Df(3R)Exel6176. Sequencing of candidate genes in the interval revealed a stop codon in the cal1 gene encoding a centromere protein (Erhardt et al. 2008; Goshima et al. 2007). This 2k32 mutant was confirmed to be a consequence of the cal1 mutation, as the mutant phenotype is complemented by a cal1 transgene (Y. Unhavaithaya and T. L. Orr-Weaver, unpublished data).

The IR8, 3C157 complementation group was mapped to 77E2-78A4 by the observation that the alleles over Df(3L)ri-XT1 caused lethality and large nuclei in the CNS. A mutation generating a stop codon was identified in the coding sequence for the kinetochore protein SPC105R in the IR8 mutant (Schittenhelm et al. 2009). Thus, we conclude that IR8 and 3C157 are alleles of Spc105R (Table 1).

This study identified new alleles of known cell-cycle regulators that affect embryogenesis. These include alleles of Cyclin E with a range of severity, and the strongest cause transformation of the cell fate of the neuroblast NB6-4. It is striking that cytokinesis functions and centromere/kinetochore proteins display embryonic lethality when disrupted. One possibility is that aspects of cell division in the nervous system make it particularly vulnerable to mitotic defects. A simpler explanation, however, is that the continued mitotic divisions that occur in the nervous system after the rest of the embryonic cells have exited the cell cycle or entered the endocycle deplete remaining maternal pools to reveal defects in the zygotic mutants.

The three PCNA in situ phenotypes linked to defects in the cell cycle or division were readily scored: arrest in the postblastoderm divisions, the presence of large cells in the nervous system, and persistence of PCNA transcripts in the endocycle domains. Thus it is notable that a small number of mutants were identified. The second chromosome screen was more exhaustive than the third, corresponding with the recovery of multiple alleles in many of the genes identified (five in Dp, four in dup, five in Cyclin E). Eight postblastoderm arrest mutants, which were not cloned, are all single-allele complementation groups. We conclude that although our screen was not saturating, a limited number of genes essential for cell division can mutate to cause embryonic defects. This most likely is explained by persistence of maternal pools through the end of embryogenesis. The alleles presented here will be a valuable resource for analysis of the mechanisms and regulation of cell division in development.

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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>cycE1056/cycE1036</td>
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*These embryos did not develop to the stage to score the neuroblast transformation. ND, not determined.

Figure 4 DAPI embryonic phenotypes. DAPI staining of the CNS and surrounding epidermis from stage 14 or 15 embryos. Homozygous embryos were distinguished from heterozygous controls by a Ubx-lacZ marker on the balancer chromosome. (A–E) Wild-type and heterozygous control embryos, stage 15. (F–I) Homozygous mutant embryos of designated genotype. DAPI staining of the embryos revealed that pavpavCS3 and tum12a-20 had cells with enlarged nuclei throughout the nerve cord and in the epidermis, whereas cal12k32 and spc105Rspc105R had isolated cells in the nervous system with increased nuclear size and ploidy (arrowheads). The pav and tum mutants arrest at stage 14; the cal1 and Spc105R are stage 15. Scale bars 20μm.
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