Ectopic Expression of the Drosophila Cdk1 Inhibitory Kinases, Wee1 and Myt1, Interferes With the Second Mitotic Wave and Disrupts Pattern Formation During Eye Development

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ABSTRACT

Wee1 kinases catalyze inhibitory phosphorylation of the mitotic regulator Cdk1, preventing mitosis during S phase and delaying it in response to DNA damage or developmental signals during G2. Unlike yeast, metazoans have two distinct Wee1-like kinases, a nuclear protein (Wee1) and a cytoplasmic protein (Myt1). We have isolated the genes encoding Drosophila Wee1 and Myt1 and are using genetic approaches to dissect their functions during normal development. Overexpression of *Dwee1* or *Dmyt1* during eye development generates a rough adult eye phenotype. The phenotype can be modified by altering the gene dosage of known regulators of the G2/M transition, suggesting that we could use these transgenic strains in modifier screens to identify potential regulators of Wee1 and Myt1. To confirm this idea, we tested a collection of deletions for loci that can modify the eye overexpression phenotypes and identified several loci as dominant modifiers. Mutations affecting the Delta/Notch signaling pathway strongly enhance a *GMR-Dmyt1* eye phenotype but do not affect a *GMR-Dwee1* eye phenotype, suggesting that Myt1 is potentially a downstream target for Notch activity during eye development. We also observed interactions with p53, which suggest that Wee1 and Myt1 activity can block apoptosis.

THE control of mitosis by inhibitory phosphorylation of cyclin-dependent kinase (Cdk)1 has been characterized extensively in unicellular eukaryotes. In *Schizosaccharomyces pombe,* signaling pathways responsive to cell size, DNA damage, and DNA replication target the phosphorylation of Cdk1 residue tyrosine 15 (Y15), thereby functioning to maintaining genome integrity (Rhind *et al.* 1997; Rhind and Russell 1998). Inhibitory phosphorylation of Cdk1 is catalyzed by both Wee1 and Mik1 kinases in *S. pombe* (Russell and Nurse 1987b; Featherstone and Russell 1991; Lundgren *et al.* 1991; Lee *et al.* 1994) and is reversed by Cdc25 and Pyp3 phosphatases (Russell and Nurse 1986; Gould *et al.* 1990; Millar *et al.* 1991, 1992). In contrast, inhibitory phosphorylation of a Cdk1 homolog (CDC28) is not required for maintenance of genome integrity in *Saccharomyces cerevisiae* (Amon *et al.* 1992; Sorger and Murray 1992). Instead, a *SWE1*-mediated checkpoint delays mitosis by inhibiting CDC28 in response to defective assembly of the actin cytoskeleton and promotes filamentous growth when nutrients are limiting (Lew and Reed 1995; Sia *et al.* 1996, 1998; McMillan *et al.* 1998; Barral *et al.* 1999; Edgington *et al.* 1999).

During Drosophila embryogenesis, inhibitory phosphorylation of Cdk1 is required for maintaining G2 phase during the embryonic cell divisions. Expression of *cdc25*^{string} overcomes this inhibition, inducing mitosis in spatially and temporally patterned mitotic domains (Edgar and O'Farrell 1990). The intricate pattern of *cdc25*^{string} transcription is governed by *cis* elements in a large regulatory region that integrates a diverse array of patterning gene inputs to direct the appropriate spatiotemporal pattern of *cdc25*^{string} expression during embryonic and imaginal development (Edgar *et al.* 1994; Johnston and Edgar 1998; Lehman *et al.* 1999). Heat shock expression of a constitutively active, nonphosphorylatable Cdk1 variant (Cdk1AF) is lethal to Drosophila embryos, indicating that inhibitory phosphorylation of Cdk1 is essential for regulating mitosis during development; however, regulation of a similar S phase kinase (Cdk2) on a conserved tyrosine residue is not (Lane *et al.* 2000).

In metazoans, two adjacent inhibitory phosphorylation sites on Cdk1 (T14 and Y15) are substrates for two distinct Wee1-like kinases that differ in their subcellular localization. Nuclear Wee1 kinases phosphorylate Y15 exclusively, whereas Myt1, a membrane-localized Wee1-like kinase, can phosphorylate either site (Kornbluth *et al.* 1994; Mueller *et al.* 1995; Booher *et al.* 1997; Liu *et al.* 1997). The physiological significance of these differences between the Wee1 and Myt1 kinases is presently unknown. We are addressing this question by characterizing the functions of Wee1 and Myt1 kinases during Drosophila development. Drosophila encodes a single *wee1* homolog (*Dwee1*), originally identified by its ability to complement a lethal mitotic catastrophe phenotype in *S. pombe* cells that were mutant for both *wee1* and *mik1*

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(Campbell *et al.* 1995). Null alleles of *Dwee1* are maternal effect lethal and *Dwee1*-derived embryos undergo catastrophic nuclear defects during the late syncytial divisions that include failure to complete nuclear division (Price *et al.* 2000) and failure to lengthen interphase, as normally occur when a developing embryo approaches cycle 14 (D. Price, unpublished data). The phenotype of *Dwee1*-derived mutant embryos is similar to phenotypes of maternal mutants for *mei-41* or *grapes* (*grp*), the Drosophila homologs of the checkpoint kinases *rad3/ATR* and *chk1,* respectively (Fogarty *et al.* 1994, 1997; Sibon *et al.* 1997, 1999). These phenotypic similarities suggest that the three genes act in a common checkpoint pathway during early embryonic development, an idea supported by genetic interactions between mutant alleles of these genes (Price *et al.* 2000).

Given the critical importance of inhibitory phosphorylation during embryogenesis, it was puzzling that the zygotic function of *Dwee1* is not essential and that *Dwee1* mutants develop normally under ordinary circumstances. *Dwee1* mutant larvae do die when they are fed hydroxyurea at concentrations that wild-type larvae can tolerate, however, apparently due to a defective DNA replication checkpoint (Price *et al.* 2000). The viability of zygotic *Dwee1* mutants could be due to the presence of a redundant Cdk1 inhibitory kinase such as Myt1. Although cellular localization and substrate specificity differences suggest that Wee1 and Myt1 homologs serve distinct roles in cell cycle regulation, the two metazoan Wee1-like kinases may also share some redundant functions, as *wee1* and *mik1* do in *S. pombe* (Lundgren *et al.* 1991). To investigate this possibility we cloned the single *Myt1*-like gene from Drosophila, *Dmyt1*, and are undertaking a genetic analysis of its function during development.

In this report we describe phenotypic defects caused by overexpressing either *Dwee1* or *Dmyt1* in developing tissues. Overexpression in the eye imaginal disc causes visible defects in the adult eye. The eye phenotype can be modified by mutations in known cell cycle regulators, suggesting that this system might be capable of detecting previously uncharacterized mitotic regulators that have evolved to coordinate cell proliferation with specific developmental events. We have tested this idea by screening for dominant genetic modifiers, using a collection of deletions comprising 70–80% of the Drosophila euchromatic genome. These tests have identified several loci that potentially encode novel regulators of either Wee1 or Myt1.

MATERIALS AND METHODS

Cloning of the Drosophila *Myt1* gene: A small fragment of *Dmyt1* was amplified by PCR using degenerate primers designed against conserved regions of Xenopus and human *Myt1* (CKLGDFG and AADVFSL). After sequencing to confirm that we had in fact isolated a genomic sequence that was similar to the *Myt1* homologs, the PCR fragment was labeled and used to screen the pNB embryonic cDNA library (Brown and Kafatos 1988). We were unsuccessful in isolating a cDNA clone by this approach, so we designed a reverse primer specific to the cloned *Dmyt1* fragment and used it in combination with a pNB vector primer to PCR amplify the 5′ end of a cDNA sequence from the same library. The fragment obtained was cloned and sequenced and the information was used to identify two cDNA clones from the Berkeley Drosophila EST Project database (GH08848 and LD34963). These clones were both fully sequenced and found to include identical coding regions that show significant sequence similarities to human and Xenopus Myt1 within the predicted kinase domain (LD34963 is 20 bp longer at the 5′ end, but the sequences are otherwise identical except for the length of the poly(A) tail at the 3′ end). The complete molecular characterization of the *Dmyt1* gene will be presented elsewhere (Z. Jin, S. Rabinovitch and S. D. Campbell, unpublished results).

Generation of *Dwee1* and *Dmyt1* transgenic stocks:

pUAST-Dwee1 and *pUAST-Dmyt1*: To synthesize *pUAST-Dwee1*, a 2.2-kb *Dwee1* cDNA fragment was excised from pBluescript SK(+) by *Kpn*I/*Not*I digestion and subcloned into the pUAST vector using the same restriction sites (Brand and Perrimon 1993). *pUAST-Dmyt1* was constructed by cloning a 1.9-kb *Eco*RI/*Xho*I fragment that includes the entire *Dmyt1* cDNA from LD34963 and inserting it into the pUAST plasmid vector, also cut with the same restriction enzymes.

pUASp-Dwee1 and *pUASp-Dmyt1*: The 2.2-kb *Kpn*I/*Not*I *Dwee1* cDNA fragment (as above) was inserted into the pUASp vector (Rørth 1998) cut with the same restriction enzymes. A PCR-amplified *Dmyt1* cDNA from the LD34963 clone containing *Kpn*I/*Not*I linker restriction sites was cloned into the pUASp vector. This clone was then sequenced to establish that no new mutations were introduced during PCR amplification.

pGMR-Dwee1 and *pGMR-Dmyt1*: The *g*lass *m*ultimer *r*eporter plasmid (pGMR; Hay *et al.* 1994) was cut with *Hpa*I and *Not*I. The *Dwee1* and *Dmyt1* cDNAs were isolated from pUASp vector constructs by cutting with *Kpn*I, blunting with T4 DNA polymerase, digestion with *Not*I, and then gel purification. Insert and vector were joined with T4 DNA ligase and the products verified by colony PCR. The transgene constructs were then injected into *y w* Drosophila embryos, using a Δ2-3-helper plasmid.

Scanning electron microscopy: Flies of the desired genotypes were collected several days after eclosion, fixed, dehydrated, and critical-point dried essentially as described in Sullivan *et al.* (2000). Critical-point-dried flies were then either imaged directly with a Philips (Cheshire, CT) ESEM (model XL30 ESEM ODP) or sputter-coated with gold and imaged with a Jeol (Tokyo) scanning electron microscope (SEM; model JSM-630FXV).

Transmission electron microscopy: Fly heads of the desired genotypes were collected, fixed, and dehydrated as described in Sullivan *et al.* (2000). Dehydrated heads were embedded in Spurr resin (Spurr 1969) with propylene oxide used as a transition solvent. Embedded heads were sectioned to ~60 nm thickness with a Diatome diamond knife using a Reichert-Jung ultramicrotome (model ULTRACUT E). Sections were collected in water on copper grids, stained with uranyl acetate and lead citrate, and viewed on a Philips transmission electron microscope (TEM; model Morgagni 268). Images were collected with a Soft Imaging System digital camera (model Megaview II).

Immunochemistry: Imaginal discs were fixed in 4% formaldehyde in PBS for 30 min at room temperature. Following fixation, the peripodial membrane was removed from the eye discs using tungsten needles. After blocking in 10% normal goat serum (NGS) made with PBS + 0.1% Tween-20 (PBT),

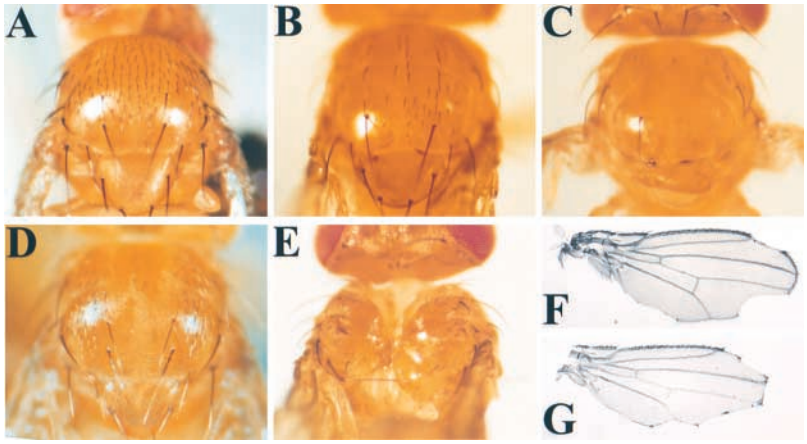

FIGURE 1.—Aberrant phenotypes caused by *Dwee1* overexpression. (A) Thorax of a wild-type fly. (B) Thorax of a fly with a single copy of *UAS-Dwee1* driven by a single copy of *ap-Gal4*. (C) Thorax of a fly with two copies of *UAS-Dwee1* and a single copy of *ap-Gal4*. (D) Thorax of a fly with a single copy of *UAS-Dwee1* and a single copy of *pnr-Gal4*. (E) Thorax of a fly with two copies of *UAS-Dwee1* and a single copy of *pnr-Gal4*. (F) Wing of a fly with a single copy of *UAS-Dwee1* and a single copy of *sd-Gal4*. (G) Wing of a fly with two copies of *UAS-Dwee1* and a single copy of *sd-Gal4*.

the fixed discs were washed three times for 5 min in PBT and incubated at 4° overnight in primary antibody (rabbit antiphosphohistone H3; Upstate Biochemicals) at 1/600 dilution in 10% NGS. Discs were then washed four times for 10 min in 5% skim milk in PBT and incubated in preabsorbed secondary antibody (goat anti-rabbit conjugated with FITC; Jackson Immunoresearch, West Grove, PA) at 1/1000 dilution. Stained discs were washed four times for 10 min in PBT, stained with Hoechst 33258, and washed again in PBT. Eye discs were then separated from the optic lobe and mounted in 80% glycerol. Images were obtained on a Zeiss (Thornwood, NY) Axioskop 2 microscope equipped with a Photometrics (Tucson, AZ) SenSys digital camera.

RESULTS

Ectopic expression of *Dwee1* in developing imaginal tissues: To examine the consequences of overexpressing *Dwee1* and *Dmyt1* in different tissues, we generated transgenic lines that can express either gene under control of the Gal4/UAS system, as described in MATERIALS AND METHODS (BRAND and PERRIMON 1993). Figure 1 shows the effect of Gal4-induced expression of *UAS-Dwee1* in various tissues (hereafter "UAS" refers to the UAST constructs). The *pannier-Gal4* (*pnr-Gal4*) and *apterous-Gal4* (*ap-Gal4*) drivers are each expressed in the developing dorsal thorax (CALLEJA *et al.* 1996). When either of these Gal4 drivers is combined with one copy of *UAS-Dwee1*, reduced numbers of sensory bristles are seen on the dorsal thorax, compared to wild type (Figure 1, A, B, and D). Flies with *ap-Gal4*-driven *UAS-Dwee1* also have upturned wings, suggesting that the dorsal compartment of the wing is smaller than the ventral compartment, consistent with these cells undergoing fewer cell divisions (data not shown). When two copies of the *UAS-Dwee1* transgene are driven by either *ap-Gal4* or *pnr-Gal4*, the bristle effects are more extreme and the dorsal epidermis is distorted, indicating that the phenotypic effects are sensitive to gene dosage (Figure 1, C and E). Combination of the *ap-Gal4* driver with two copies of *UAS-Dwee1* yields a nearly bald dorsal thorax accompanied by a severe reduction of the scutellum (Figure 1C). A more extreme phenotype is seen when the *pnr-Gal4* driver is combined with two copies of *UAS-Dwee1*, producing a furrowed thorax, as if the two halves have failed to fuse properly (Figure 1E). This observation suggests that fusion may require temporally or spatially regulated cell divisions that can be blocked by our overexpression system. In the wing, *UAS-Dwee1* combined with a wing-specific *sd-Gal4* driver line produces extensive scalloping of the wing margin (Figure 1F) and an additional copy of *UAS-Dwee1* (Figure 1G) also increases the severity of this mutant phenotype.

Ectopic *Dwee1* expression in the eye produces a rough eye phenotype (Figure 2). In Figure 2, A and B, are controls showing a wild-type eye and an eye from a fly with a single copy of the *ninaE-Gal4* driver, respectively (FREEMAN 1996). When *UAS-Dwee1* is combined with the *ninaE-Gal4* driver, the eye facets are disorganized and frequent duplications of bristles are observed (Figure 2C). *ninaE-Gal4* overexpression of *Dmyt1* produced a similar phenotype (not shown). The *Dwee1* and *Dmyt1*-induced rough eye phenotypes suggested to us that we could use *Dwee1* or *Dmyt1* transgenic flies in an assay system for identifying negative or positive regulators of mitosis, as described below.

Genetic interactions with *GMR-Dwee1* and *GMR-Dmyt1*: The GMR overexpression vector uses a Glass transcription factor-binding enhancer to direct transgene expression posterior to the morphogenetic furrow (MF) in the developing eye (HAY *et al.* 1994). This single component system thus provides a convenient tool for rapidly testing genetic interactions. After cloning the cDNAs for each gene into this vector, we observed that *GMR-Dwee1* and *GMR-Dmyt1* transgenic lines each show dosage-sensitive rough eye phenotypes. In ~12 independent transgene lines examined for each construct, the *Dmyt1*-induced phenotypes are consistently stronger than the *Dwee1*-induced phenotypes, suggesting a stronger effect of Myt1 on eye development that is not attributable to chromosomal position effects (data not shown). In Figure 3B we show an adult eye from a fly carrying four

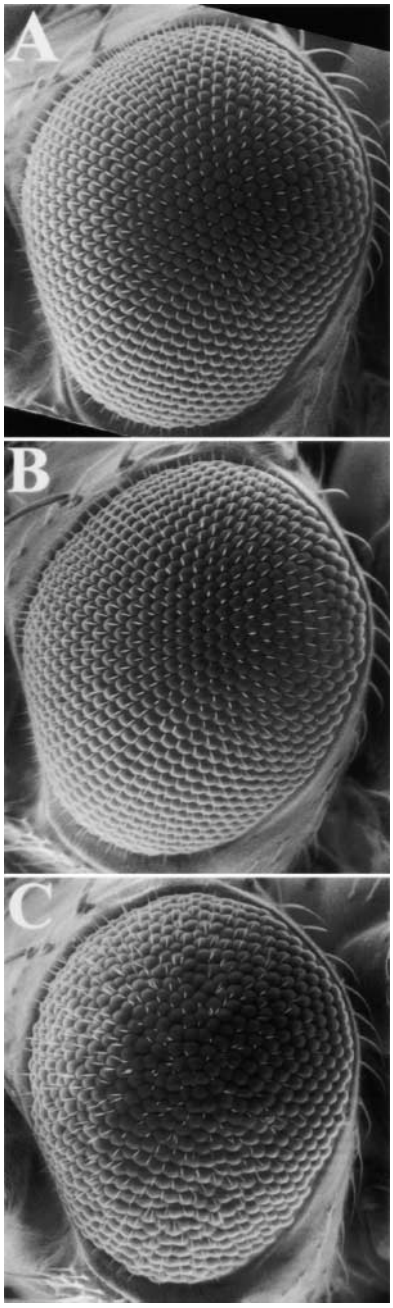

Figure 2.—Effects of *Dwee1* overexpression on the adult eye as visualized by SEM. (A) A single copy of the *ninaE-Gal4* driver transgene. (B) A single copy of the *UAS-Dwee1* transgene. (C) A single copy of *UAS-Dwee1* driven by a single copy of the *ninaE-Gal4* transgene.

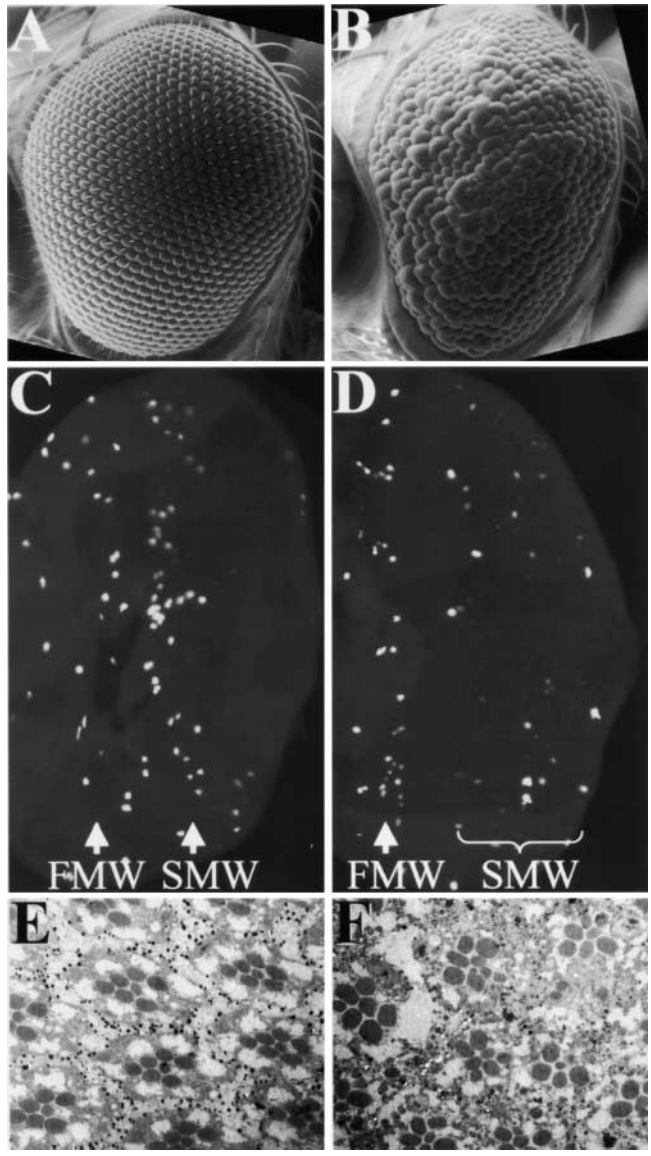

Figure 3.—Effects of *Dmyt1* overexpression in the developing and adult eye. (A) SEM of an eye from a wild-type fly. (B) SEM of an eye from a fly with four copies of *GMR-Dmyt1*. (C) Eye-antennal disc from a wild-type fly stained with the mitotic marker, antiphosphohistone H3 (αPH3), showing mitotic figures in the first (FMW) and second (SMW) mitotic waves. (D) αPH3-stained eye-antennal disc from a fly with four copies of *GMR-Dmyt1*. The SMW is disrupted and delayed, as shown by the decreased number and increased spread of mitotic figures posterior to the FMW. (E) TEM cross section of an adult eye from a wild-type fly. (F) TEM cross section of an adult eye from a fly with four copies of *GMR-Dmyt1*.

copies of *GMR-Dmyt1,* compared with a wild-type control eye (Figure 3A). Posterior to the MF, the second mitotic wave (SMW) generates a pool of uncommitted cells for recruitment into the developing ommatidial preclusters (Wolff and Ready 1991). To test our assumption that the aberrant phenotypes we observe when Wee1 or Myt1 are overexpressed are a consequence of inhibiting or delaying cell divisions required for normal development, we examined mitotic activity in eye imaginal discs isolated from a *GMR-Dmyt1* transgenic strain. Figure 3C shows mitotic activity in a wild-type third larval instar eye disc, visualized by antibody staining for phosphohistone H3. In discs isolated from a *GMR-Dmyt1* transgenic line, mitoses in the SMW are both reduced in number and delayed (inferred from the increased distance of mitotic cells from cells of the "first mitotic wave"; Figure 3D) when compared to wild type. Mitoses ahead of the mor-

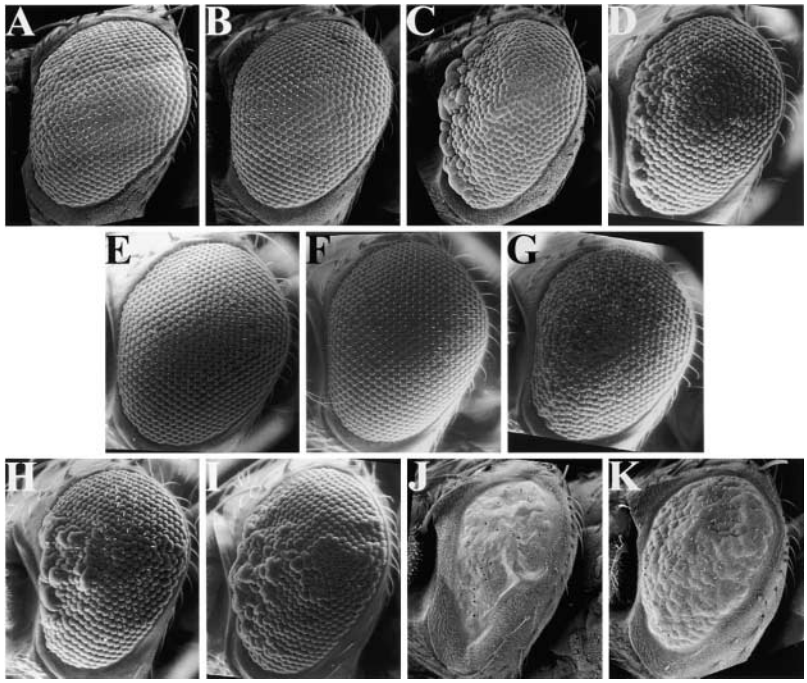

Figure 4.—SEM analysis of eye phenotypes seen in genetic interactions with *GMR-Dwee1* and *GMR-Dmyt1*. (A) SEM showing a fly with a single copy of *GMR-Dmyt1*. (B) Fly heterozygous for a mutation in the $cdc25^{string}$ locus. (C) Fly with a single copy of *GMR-Dmyt1* and heterozygous for a mutation in the $cdc25^{string}$ locus. (D) Fly with a single copy of *GMR-Dmyt1* and heterozygous for a mutation in the *cdc2* locus. (E) Fly with a single copy of *GMR-Dwee1*. (F) Fly heterozygous for a mutation in the *cdc2* locus. (G) Fly with a single copy of *GMR-Dwee1* and heterozygous for a mutation in the *cdc2* locus. (H) Fly with a single copy of *GMR-rux*. (I) Fly with single copies of both *GMR-Dmyt1* and *GMR-rux*. (J) Fly with a single copy of *p53-pExP-glass*. (K) Fly with single copies of both *GMR-Dmyt1* and *p53-pExP-glass*.

phogenetic furrow (the first mitotic wave) are unaffected by *GMR-Dmyt1*, as expected since *GMR*-driven expression does not occur in this region of the disc (Hay *et al.* 1994). We also observed that the ommatidial preclusters in the *GMR-Dmyt1* flies appear disorganized when visualized by transmission electron microscopy of sectioned adult eyes. Figure 3, E and F, shows the effects of *GMR-Dmyt1* on the arrangement of photoreceptor cells. Most of the identifiable cell types in the ommatidia appear to be present, although the arrangement and size of the rhabdomeres are often irregular. The *GMR-Dmyt1* photoreceptor cell clusters often contain too few or too many cells, however, and there is a striking disruption of the regular hexagonal array of secondary and tertiary pigment cells that normally forms an interface between adjacent ommatidia (compare Figure 3E and 3F).

We next tested for genetic interactions with a set of cell cycle regulatory mutants that are predicted to either have a direct regulatory interaction with *Dwee1* or *Dmyt1* or play an independent role in Cdk1 regulation. Mutations in factors that normally promote the onset of mitosis should enhance the *Dwee1* or *Dmyt1* overexpression phenotypes, whereas mutations in genes that function to delay mitosis should show the reverse effect. Figure 4 illustrates several such interactions. A single transgene copy of *GMR-Dmyt1* produces a mild rough eye phenotype, whereas independently, a heterozygous mutation in $cdc25^{string}$ has no effect on eye morphology (Figure 4, A and B). When a single copy of *GMR-Dmyt1* is combined with a heterozygous mutation for $cdc25^{string}$, a significantly enhanced eye phenotype is seen (Figure 4C). Likewise, removal of a single copy of *cdc2* produces a similar effect in combination with a single copy of *GMR-Dmyt1* (Figure 4D). The *GMR-Dmyt1/*$cdc25^{string}$ interaction produces an eye that is devoid of bristles, whereas the *GMR-Dmyt1/cdc2* interaction shows milder bristle effects. Curiously, the dominant enhancement seen in these cases is consistently stronger in more anterior parts of the eye that differentiate later in development. Cdc2 (now called Cdk1) and its activating phosphatase, $Cdc25^{string}$ are essential for promoting mitosis in Drosophila (Edgar and O'Farrell 1989; Stern *et al.* 1993), so these genetic interactions are consistent with known functions for these genes. A weak single-copy *GMR-Dwee1* phenotype (Figure 4E) is also enhanced by heterozygous mutant alleles of *cdc2* (Figure 4G), but unlike *GMR-Dmyt1*, not by heterozygous mutations for $cdc25^{string}$ (not shown). These genetic interactions were confirmed with multiple alleles of *cdc2* and $cdc25^{string}$ to rule out nonspecific genetic background effects. We also tested a number of other known cell cycle mutants for dominant modifier effects on either *GMR-Dwee1* or *GMR-Dmyt* phenotypes. Mutations in *cyclin A*, *cyclin B*, *mei-41*, *grapes*, *twine*, *cdk2*, *cyclin E*, *fizzy*, and *dacapo* all fail to either enhance or suppress the rough eye phenotype generated by either transgene.

The *rux* gene encodes a cyclin-dependent kinase inhibitor (CKI) that inhibits Cyclin A/Cdk1 by promoting the degradation of cyclin A (Thomas *et al.* 1994, 1997; Sprenger *et al.* 1997; Foley *et al.* 1999; Avedisov *et al.* 2000). When *GMR-Dmyt1* (Figure 4I) or *GMR-Dwee1* (not shown) is coexpressed with *GMR-roughex* (*GMR-rux*) the phenotype is enhanced relative to that generated by *GMR-rux* alone (Figure 4H), resulting in a stronger rough eye phenotype that is accompanied by a near complete loss of bristles. While this result is consistent with addi-

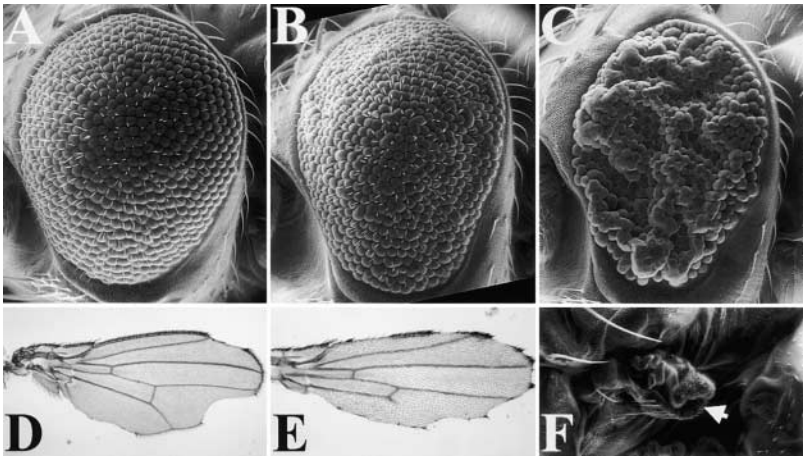

FIGURE 5.—Coexpression of *Dwee1* and *trbl* shows strong synergistic phenotypic effects. (A) SEM of a fly with one copy of *UAS-Dwee1* driven by one copy of *ninaE-Gal4*. (B) Fly with one copy of *UAS-trbl* driven by one copy of *ninaE-Gal4*. (C) Fly with single copies of both *UAS-Dwee1* and *UAS-trbl* driven by a single copy *of ninaE-Gal4*. (D) Wing of a fly with one copy of *UAS-Dwee1* driven by *sd-Gal4* (hemizygous on the X chromosome). (E) Wing of a fly with one copy of *UAS-trbl* driven by *sd-Gal4*. (F) Fly with single copies of both *UAS-Dwee1* and *UAS-trbl* driven by *sd-Gal4*. The arrowhead indicates the position of the small piece of wing tissue.

tive effects of these Cdk1 inhibitors, we also made the surprising observation that otherwise viable zygotic *Dwee1* mutants show near-complete synthetic lethality with otherwise viable zygotic *rux* mutants. Rare double-mutant "escapers" from these genetic crosses show various phenotypic abnormalities, including enhancement of the *rux* rough-eye phenotype, bristle duplications and deletions, and "Minute" bristles (data not shown).

To investigate genetic interactions with a known component of the DNA damage response pathway, we tested the Drosophila homolog of the *p53* tumor suppressor gene. Expression of a *p53-pExP-glass* transgene promotes apoptosis, generating eye tissue that has no evidence of intact ommatidia or bristles (OLLMANN *et al.* 2000; Figure 4J). Coexpression of a single transgene copy of either *GMR-Dmyt1* (Figure 4K) or *GMR-Dwee1* (not shown) can markedly suppress this phenotype, with recovery of the eye bristles being most pronounced (compare Figure 4J with 4K).

The *tribbles* (*trbl*) gene encodes a novel mitotic inhibitor that functions in mesodermal cells during early gastrulation (GROSSHANS and WIESCHAUS 2000; MATA *et al.* 2000; SEHER and LEPTIN 2000). *ninaE-Gal4*-driven *UAS-Dwee1* or *UAS-trbl* transgenes alone generate slightly roughened eyes, with occasional duplication of bristles (Figure 5, A and B). When the two genes are coexpressed in the eye, the ommatidial phenotype is dramatically enhanced and there is a near complete loss of bristles (Figure 5C). In a complementary experiment, the eye phenotype generated by two copies of *GMR-Dmyt1* combined with a single copy of *GMR-Dwee1* is partially suppressed by removal of one gene copy of *trbl* (data not shown). These striking synergistic interactions are not confined to eye development, as coexpression of *UAS-Dwee1* and *UAS-trbl* yields nearly complete ablation of wing tissue (Figure 5F), compared with scalloping of the wing margin observed when *UAS-Dwee1* or *UAS-trbl* are expressed singly with the *sd-Gal4* driver (Figure 5, D and E). Occasional conversions of wing tissue to apparent thoracic tissue were also noted in these coexpression experiments. Unlike the similar wing margin phenotypes we observe when *UAS-trbl* or *UAS-Dwee1* are expressed during wing development, *UAS-trbl* expression is associated with a noticeable reduction of trichome density in the wing blade that apparently reflects increased cell size, a phenotype that is not observed with *UAS-Dwee1* (compare Figure 5D and 5E).

We next conducted genome-wide screens for loci that modify *GMR-Dwee1* or *GMR-Dmyt1* eye phenotypes, using the Drosophila deficiency kit (maintained by the Bloomington Drosophila Stock Center). The kit presently comprises 195 stocks that are estimated to cover 70–80% of the Drosophila euchromatic genome. In two separate screens, we tested these deletions for their ability to enhance the eye phenotypes associated with single-copy transgenic stocks of either *GMR-Dmyt1* or *GMR-Dwee1*. In a third screen to identify both enhancer and suppressor loci, we tested the deletions against a stock carrying two copies of *GMR-Dmyt1* and one copy of *GMR-Dwee1* (made by recombination of different transgene insertions). The genetic crosses were scored without reference to whether or not the deletions uncovered any known cell cycle regulators, to avoid biasing our results. The genetic loci that we have identified in these screens, as cytological regions defined either by deletions or by mutations in specific genes, are compiled in Table 1. Consistent with observations based on single alleles, *Df(2L)Mdh*, which includes the *cdc2* locus, enhances the phenotype of all three tester strains, whereas deletions that include $cdc25^{string}$ [*Df(3R)3450* and *Df(3R)Dr-rv1*] were selected as enhancers of *GMR-Dmyt1* and *2xGMR-Dmyt1, 1xGMR-Dwee1* in this assay, but not as enhancers of the *GMR-Dwee1* transgene alone.

Six deletions, four of which represent loci not previously identified in crosses with known cell cycle regulators, were identified as specific enhancers of *GMR-Dmyt1* (Table 1). One of the *GMR-Dmyt1* enhancer regions [*Df(3R)Dl-BX12*] contains *Delta* (*Dl*), which encodes a ligand for signaling through the Notch pathway. Independent tests with specific alleles of *Dl* have confirmed

TABLE 1

Summary of interacting mutations/deficiencies

Test stock	Enhancement	Suppression
GMR-Dmyt1	*cdc*	
	cdc25^{string}	
	Delta	
	Df(2L)net-PMF (021A01;021B07-08)	
	Df(2L)Mdh (030D-30F;031F)	
	Df(2L)r10 (035D01;036A06-07)	
	Df(3L)pbl-X1 (065F03;066B10)	
	Df(3R)Dl-BX12 (091F01-02;092D03-06)	
	Df(3R)3450 (098E03;099A06-08)	
	Df(3R)Dr-rv1 (099A01-02;099B06-11)	
GMR-Dwee1	*cdc2*	
	Df(2L)Mdh (030D-30F;031F)	
	Df(3R)e-R1 (093B06-07;093D02)	
2xGMR-Dmyt1,	*cdc2*	*Notch*
1xGMR-Dwee1	*cdc25*^{string}	*tribbles*
	Delta	*Df(1)N-8* (003C02-03;003E03-04)
	Df(2L)net-PMF (021A01;021B07-08)	*Df(3L)st4* (072D10;073C01)
	Df(2L)MDh (030D-30F;031F)	
	Df(2L)r10 (035D01;036A06-07)	
	Df(2R)vg-C (049A04-13;049E07-F01)	
	Df(3R)Dl-BX12 (091F01-02;092D03-06)	
	Df(3R)3450 (098E03;099A06-08)	
	Df(3R)Dr-rv1 (099A01-02;099B06-11)	

that *Dl* is the gene responsible for this interaction. Since some alleles of *Dl* exhibit dominant eye phenotypes (specifically, Dl^{1}), it is important to note that we observed enhancement with alleles (Dl^{3}, Dl^{7}, Dl^{B2}, and Dl^{RevF10}) that by themselves are not associated with a dominant eye phenotype. It is unlikely, therefore, that these interactions reflect additive effects. We saw similar enhancement with gene duplications of the *Notch* locus, which on their own are associated with a "Confluens" or *Delta*-like phenotype [*Dp(1;2)51b*, $Dp(1;2;Y)w^{+}$, and *Dp(1;2)72c21*]. A deletion of the Notch locus, on the other hand [*Df(1)N-8*], suppresses the phenotype associated with a *2xGMR-Dmyt1, 1xGMR-Dwee1* strain. Specific genes responsible for the remaining three *GMR-Dmyt1* enhancer interactions have not yet been identified. *Df(2L)r10* contains three known mitotic regulatory genes (*grapes*, *twine*, and *fizzy*), none of which behaves as an enhancer in tests with specific mutant alleles, however. It is possible that the phenotypic modification seen with this deletion reflects a combinatorial interaction with more than one of these genes.

Only two cytological regions, identified by crosses to the deletion collection, were identified as specific enhancers of a *GMR-Dwee1* eye phenotype, one of which contains *cdc2* (Table 1). We have not yet identified the gene responsible for the remaining suppressor interaction with *2xGMR-Dmyt1, 1xGMR-Dwee1* that is associated with *Df(3L)st4*. Further analysis to identify and characterize the remaining gene modifiers will now be necessary to determine if these loci do in fact encode distinct regulators for *Dwee1* and *Dmyt1*.

DISCUSSION

The G1/S and G2/M cell cycle transitions are temporally and spatially controlled during metazoan development, allowing growth and cell division to be coordinated with patterning and differentiation (reviewed by Edgar and Lehner 1996). Studies of G2/M checkpoint controls in metazoans have emphasized regulatory mechanisms affecting the Cdc25-like phosphatases, which activate the mitotic regulator Cdk1 by removing inhibitory phosphorylation. Regulatory mechanisms affecting the activity and protein stability of the Cdk1 inhibitory kinases are still poorly understood, but are probably just as important (Michael and Newport 1998; Lee *et al.* 2001). There are ample precedents for these mechanisms from studies of Wee1 and Mik1 kinases in *S. pombe* (Russell and Nurse 1987a; Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993; O'Connell *et al.* 1997; Raleigh and O'Connell 2000) and SWE1 in *S. cerevisiae* (Lew and Reed 1995; Sia *et al.* 1996, 1998; Barral *et al.* 1999; Edgington *et al.* 1999; McMillan *et al.* 1999).

During the third larval instar, the Drosophila eye disc undergoes progressive transformation from a relatively amorphous epithelial sac into the complex arrangement of ommatidial facets that comprises the adult compound eye. This transformation is marked by passage of a constriction called the MF across the eye disc (Wolff and Ready 1991). Cells within the MF normally arrest in G1 and failure to synchronize cells at this stage disrupts ommatidial patterning (Thomas *et al.* 1994). Following the MF, a population of cells called the SMW undergoes a final cell cycle. If cells are blocked in G1 by overexpression of a p21 CKI homolog, insufficient cells are left to form all of the cell types required for normal ommatidia, resulting in a rough adult eye phenotype (de Nooij and Hariharan 1995; de Nooij *et al.* 1996). In this report, we have shown that *GMR*-driven misexpression of *Dmyt1* immediately after the MF both delays the SMW divisions and reduces the numbers of mitotic cells, also resulting in a rough eye phenotype.

We have established that *Dwee1* and *Dmyt1* overexpression eye phenotypes are sensitive to modification by mutations in known cell cycle regulatory genes, illustrating the feasibility of screening for mutations of genes that are potential regulators of either Wee1 or Myt1. Mutations in genes that promote mitosis, such as *cdc2* and $cdc25^{string}$, should dominantly enhance these overexpression phenotypes and we have confirmed this expectation for both of these genes with *Dmyt1*. Although a *GMR-Dwee1* eye phenotype is also enhanced by mutations in *cdc2*, it is not enhanced by mutations in $cdc25^{string}$, providing evidence that Wee1 and Myt1 kinases have distinct Cdk1 regulatory effects in this developmental context. This result could be explained by a requirement for higher levels of $cdc25^{string}$ activity to overcome *GMR-Dmyt1* inhibition of Cdk1 relative to *GMR-Dwee1*, perhaps because it is inherently more difficult to dephosphorylate Cdk1 inhibited on both T14 and Y15 by Myt1 activity, compared with Cdk1 inhibited on Y15 alone by Wee1.

The *rux* gene encodes a novel Cdk1 inhibitor that controls the onset of S phase during embryogenesis, eye development, and spermatogenesis (Gonczy *et al.* 1994; Thomas *et al.* 1994, 1997; Sprenger *et al.* 1997; Foley *et al.* 1999; Avedisov *et al.* 2000). A recent study has shown that *rux* also plays a novel role in mitosis, by an unknown mechanism (Foley and Sprenger 2001). Rux and Wee1 both negatively regulate Cdk1 activity; thus our observation that coexpression of these genes generates more extreme rough eye phenotypes than seen with either alone is consistent with known functions for these genes. Surprisingly, we also found that flies lacking both zygotic *Dwee1* and *rux* functions show nearly complete synthetic lethality, with rare escapers exhibiting extensive adult bristle phenotypes. This interaction suggests that *rux* and *Dwee1* may also cooperate in some other, as yet undefined regulatory mechanism. The extensive bristle phenotypes seen in *rux ; Dwee1* double mutant escapers could indicate disruption of cell cycle timing or abrogation of genome integrity checkpoints, similar to the phenotypes seen in *mus304* mutants exposed to ionizing radiation, which are associated with increased genome instability (Brodsky *et al.* 2000). Another piece of evidence suggesting a role for Wee1 kinases in regulating genome stability is the interaction we observe with Drosophila *p53*. In humans, the p53 tumor suppressor promotes apoptosis in cells that have suffered DNA damage. Overexpression of Drosophila *p53* in the eye promotes extensive cell death by apoptosis, resulting in extremely defective eyes (Ollmann *et al.* 2000). We have shown significant suppression of the *p53* overexpression eye phenotype by coexpression of either *GMR-Dwee1* or *GMR-Dmyt1*, suggesting that these Cdk1 inhibitory kinases can negatively regulate p53-induced apoptosis. Since Cdk1 activity has previously been implicated in promoting apoptosis, this effect would be consistent with known functions of Wee1 and Myt1 in Cdk1 inhibition (Zhou *et al.* 1998). Other reports relevant to this issue are somewhat contradictory, however. In human cell culture, Wee1 can inhibit granzyme B-induced apoptosis; furthermore, Wee1 appears to be downregulated through a p53-dependent mechanism, suggesting that p53 regulation of Wee1 might normally occur during this process (Chen *et al.* 1995; Leach *et al.* 1998). In contrast, Smith *et al.* (2000) showed that Wee1 activity can actually promote apoptosis in a Xenopus oocyte extract system. Further studies are clearly needed to establish the physiological significance of any purported roles for Wee1 or Myt1 in regulating apoptosis, p53-dependent or otherwise.

A screen for modulators of *wee1* overexpression was previously conducted in *S. pombe*, by isolating suppressors of *wee1*-induced lethality (Aligue *et al.* 1994; Munoz and Jimenez 1999; Munoz *et al.* 1999). These studies identified mutations in the gene encoding the Hsp90 chaperone as potent suppressors, suggesting a role for Hsp90 in promoting the assembly and/or disassembly of functional Wee1 protein complexes. In contrast, we have not found *hsp83* mutant alleles (encoding Drosophila Hsp90) to act as suppressors of a combined *GMR-Dmyt1/GMR-Dwee1* transgene eye phenotype (data not shown). We have, however, identified several other genetic loci as specific enhancers of eye phenotypes generated by *GMR-Dwee1* or *GMR-Dmyt1* alone, indicating that phenotypic effects mediated by Wee1 and Myt1 are responsive to lowered expression of different genes. These observations may reflect differences in threshold requirements for the relevant gene products in promoting mitosis (as suggested by the interactions with $cdc25^{string}$) or they may signify differences in the regulation of Wee1 and Myt1 kinases that we will now be able to dissect by identifying and characterizing the relevant modifier loci. We are currently undertaking direct genetic screens for mutations in genes that modify *GMR-Dwee1* and *GMR-Myt1* eye phenotypes to address this

issue. One of the loci we have identified as a specific enhancer of the *GMR-Dmyt1* eye phenotype is *Delta*. This interaction could reflect defects in *Dl*-dependent neuronal specification that are enhanced by *GMR-Dmyt1* activity, or it may indicate a novel role for Delta/Notch signaling in regulating Myt1 activity. We are presently trying to distinguish these possibilities.

In *S. pombe*, the DNA damage and DNA replication checkpoint pathways that regulate Cdk1 by inhibitory phosphorylation act by controlling the activity and stability of Wee1 and Mik1 kinases, as well as Cdc25 phosphatases (reviewed by Walworth 2000). Although metazoan homologs of components of these checkpoint pathways show significant sequence conservation with their yeast homologs, the actual functions and interactions of individual components are not necessarily conserved. For example, Guo and Dunphy (2000) showed that Xenopus homologs of the checkpoint kinases Chk1 and Cds1, which respond to DNA damage and blocked DNA replication, respectively, in *S. pombe*, respond in the exact opposite manner to these stresses in Xenopus egg extracts. This example serves as a warning that simple predictions of metazoan gene function based on extrapolation from known functions of yeast genes can be misleading. Metazoan development requires that novel regulatory mechanisms exist to link specific developmental processes with the basic cell cycle machinery. Drosophila represents an ideal model for analyzing these developmental controls of the cell cycle, since the effects of specific mutations on complex processes like morphogenesis and differentiation can be established. The recent characterization of the *trbl* gene in Drosophila illustrates this point (Grosshans and Wieschaus 2000; Mata *et al.* 2000; Seher and Leptin 2000). Trbl activity delays mitosis in invaginating G2 cells (mitotic domain 10) in a cycle 14 embryo. Although *cdc25*^{string} transcription initiates in domain 10 before it is transcribed in other cells, these cells remain G2 arrested until they are completely internalized, well after cells in nine other mitotic domains have subsequently expressed *cdc25*^{string} and entered mitosis (Edgar and O'Farrell 1989). Trbl activity downregulates Cdc25^{string} protein stability, providing an explanation for these observations (Mata *et al.* 2000). A similar purpose could be served by Trbl simultaneously upregulating *Dwee1* or *Dmyt1* activity (Grosshans and Wieschaus 2000). Intriguingly, Trbl contains motifs reminiscent of Nim1-type kinases, which negatively regulate Wee1 and Swe1 kinase activity and stability in *S. pombe* and *S. cerevisiae* (Russell and Nurse 1987a; Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993; Barral *et al.* 1999). Despite these sequence similarities, the Trbl protein apparently lacks a functional catalytic domain, raising the possibility that Trbl could act in a "dominant negative" manner to activate Wee1 (or Myt1) by interfering with the activities of Nim1-like inhibitors. Genetic interactions that we describe in this report are consistent with this possibility.

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