

Dominant mutations in the *Caenorhabditis elegans* Myt1 ortholog *wee-1.3* reveal a novel domain that controls M-phase entry during spermatogenesis

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Accepted 12 July 2002

SUMMARY

Regulatory phosphorylation of the Cdc2p kinase by Wee1p-type kinases prevents eukaryotic cells from entering mitosis or meiosis at an inappropriate time. The canonical Wee1p kinase is a soluble protein that functions in the eukaryotic nucleus. All metazoa also have a membrane-associated Wee1p-like kinase named Myt1, and we describe the first genetic characterization of this less well-studied kinase. The *Caenorhabditis elegans* Myt1 ortholog is encoded by the *wee-1.3* gene, and six dominant missense mutants prevent primary spermatocytes from entering M phase but do not affect either oocyte meiosis or any mitotic division. These six dominant *wee-1.3(gf)* mutations are located in a four amino acid region near the C terminus and they cause self-sterility of hermaphrodites. Second-site intragenic suppressor mutations in *wee-1.3(gf)* restore self-

fertility to these dominant sterile hermaphrodites, permitting genetic dissection of this kinase. Ten intragenic *wee-1.3* suppressor mutations were recovered and they form an allelic series that includes semi-dominant, hypomorphic and null mutations. These mutants reveal that WEE-1.3 protein is required for embryonic development, germline proliferation and initiation of meiosis during spermatogenesis. This suggests that a novel, sperm-specific pathway negatively regulates WEE-1.3 to allow the G2/M transition of male meiosis I, and that dominant *wee-1.3* mutants prevent this negative regulation.

Key words: Cell cycle, Meiosis, Wee1p, Myt1, Spermatogenesis, *C. elegans*

INTRODUCTION

The eukaryotic cell division cycle coordinates cell growth with chromosomal replication so that two daughter cells containing all required components form at the appropriate time (Nurse, 2000). This process is divided into phases of gap 1 (G1 phase), DNA synthesis (S phase), a second gap (G2 phase) and mitosis (M-phase). Transit through the eukaryotic cell cycle is governed by evolutionarily conserved cyclin-dependent kinases (CDKs) that are tightly regulated by numerous kinases, phosphatases and small inhibitory proteins (Pavletich, 1999). Yeast use a single mitotic CDK and multiple cyclins to control cell cycle progression (Stern and Nurse, 1996). However, higher eukaryotes possess multiple mitotic CDKs (Nigg, 1995) and each mitotic CDK associates with a specific cyclin that can vary with the cell cycle stage (Shulman, 1998). Successive activation of multiple CDK/cyclin complexes is thought to promote cell cycle progression in higher eukaryotes.

Mitotic entry is controlled by activation of a protein complex that is composed of the Cdc2p kinase and cyclin B (Dunphy et al., 1988). This protein complex accumulates during late G2 phase, but phosphorylation of threonine-14 and tyrosine-15 on Cdc2p prevents its activation (Coleman and Dunphy, 1994).

Dephosphorylation of these residues constitutes the major mitotic entry signal in eukaryotes. In fission yeast, Y15 of Cdc2p is phosphorylated by the Wee1p and Mik1p kinases (Lundgren et al., 1991), while dephosphorylation is carried out by the Cdc25p phosphatase (Russell and Nurse, 1986; Berry and Gould, 1996; Gautier et al., 1991). In metazoans, T14 phosphorylation is catalyzed exclusively by a member of the Wee1p kinase family called Myt1 (Fattaey and Booher, 1997). The Myt1 kinase can also phosphorylate Y15 of Cdc2p, is about 40% identical and 70% similar to the canonical *S. pombe* Wee1p kinases in its kinase domain, contains a predicted transmembrane domain, and has a C-terminal domain of poorly understood function. The membrane-spanning domain mediates Myt1 endoplasmic reticulum and Golgi localization in human cell culture lines when this protein is overexpressed (Liu et al., 1997). Thus, Myt1 is a distinct member of the Wee1p kinase family that appears to function in the cytoplasm to regulate Cdc2p.

We have analyzed rare dominant mutants that affect *C. elegans* spermatogenesis (*spe* mutants) and show that they contain mutations in the *C. elegans wee-1.3* gene. The *wee-1.3* gene is the *C. elegans* Myt1 ortholog and these are the first Myt1 kinase mutations recovered in any organism. These

dominant *spe* mutants cannot perform the G2/M transition during spermatogenesis so hermaphrodites lack mature spermatozoa and exhibit, consequently, self-sterility. Genetic suppression of this self-sterility allowed recovery of loss-of-function mutants in *C. elegans wee-1.3*. These loss-of-function mutations reveal that WEE-1.3 is required for *C. elegans* embryonic and larval development. Sequence analysis of both dominant and intragenic suppressor lesions show that all dominant mutations affect a small domain in the *C. elegans* WEE-1.3 C-terminal region, while null suppressors affect the kinase domain. This suggests that *C. elegans* WEE-1.3 kinase activity is inhibited during M phase of meiosis I during spermatogenesis by a tissue-specific regulatory mechanism.

MATERIALS AND METHODS

Strains, culture and nomenclature

Nematode culture and handling were performed as previously described (Brenner, 1974). All strains are derived from the wild-type N2 strain (var. Bristol), and all animals were maintained at 20°C unless otherwise noted. The following genetic markers and balancers were used.

LG II: *dpy-2(e8)*, *dpy-10(e128)*, *unc-4(e120)* (Brenner, 1974); *mab-3(e1240)*, *eDf21* (Shen and Hodgkin, 1988); *mIn1[dpy-10(e128) mIs14]* (Edgley and Riddle, 2001); *mnC1[dpy-10(e128) unc-52(e444)]*, *let-241(mn228)*, *mnDf12*, *mnDf28*, *mnDf29*, *mnDf30*, *mnDf57*, *mnDf58*, *mnDf60*, *mnDf63*, *mnDf71* and *mnDf88* (Sigurdson et al., 1984).

LG III: *dpy-1(e1)* (Brenner, 1974) and *smg-6(r896)* (Hodgkin et al., 1989).

LG IV: *dpy-20(e1282ts)* (Hosono, 1982).

mIs14 confers a dominant GFP+ phenotype in pharyngeal muscle, gut, and in four- to 60-cell embryos, but only the pharyngeal marker was used during these studies (Edgley and Riddle, 2001).

All dominant *spe* mutants were recovered following EMS mutagenesis under standard conditions (Brenner, 1974) and each bears a mutation in a gene initially called *spe-37*, but now named *wee-1.3* for nomenclatural clarity. *wee-1.3(e1947)* was isolated as previously described (Doniach, 1986). *wee-1.3(q89)* was isolated in the laboratory of Judith Kimble and provided by T. Schedl. *wee-1.3(hc144)* and *wee-1.3(hc145)* were isolated by J. Varkey in the laboratory of Sam Ward. *wee-1.3(eb95)* was isolated in a F₁ non-complementation screen for new recessive *spe-10* alleles (W. Lindsey and S. W. L., unpublished). *wee-1.3(eb104)* was isolated in a F₁ non-complementation screen for new recessive *fer-14* alleles (T. Kroft and S. W. L., unpublished).

wee-1.3 suppression genetics

wee-1.3(gf) was balanced by mating *wee-1.3(gf) unc-4/ dpy-2 unc-4* hermaphrodites to *dpy-2 unc-4/mnC1* or *mIn1* males and picking F₁ Uncs to verify the Spe phenotype. Either γ -ray or ENU mutagenesis (Anderson, 1995) was performed prior to screening for suppression of the dominant Spe phenotype. It was necessary to devise a mating scheme to accumulate sufficient animals for mutagenesis because they were dominant Spe. For ENU mutagenesis, one *wee-1.3(q89gf) unc-4/mnC1* hermaphrodite was mated to four *dpy-2 unc-4/mIn1* GFP+ males on each of over 100 plates. The animals on each mate plate were transferred daily for 4 days so that the outcross progeny on each plate were of a similar age. When many L4 F₁ were present, they were pooled from the mate plates and mutagenized with 3.125 mM ENU for 4 hours under conditions similar to those described for ethylmethane sulfonate (Brenner, 1974). Mutagenized GFP+ nonUnc hermaphrodites (~300-350) were picked to individual plates to verify that these putative *wee-1.3(gf) unc-4/mIn1*GFP+ showed the expected

(unfertilized oocyte laying) Spe phenotype. Each verified self-sterile Po hermaphrodite was mated to four *dpy-2 unc-4/mnC1* males and 6968 resulting F₁ Unc progeny were each picked to separate plates. Self-fertile *wee-1.3(q89;sup) unc-4/dpy-2 unc-4* hermaphrodites that segregated nonGFP Uncs and DpyUncs in the F₂ were selected as candidate suppressors. They were outcrossed to *dpy-2 unc-4/mIn1* GFP+ males and F₁ GFP+ nonUnc progeny were picked. Balanced *wee-1.3(gf;sup) unc-4/mIn1* GFP+ were identified as hermaphrodites that failed to segregate DpyUnc progeny.

For γ -ray mutagenesis, L4 hermaphrodites of genotype *dpy-2 wee-1.3(e1947)/dpy-2 unc-4* were mutagenized with 1500 Rads from a ¹³⁷Cs source. These hermaphrodites were crossed to *dpy-2 unc-4/mnC1* males and 125 Dpy outcross progeny were each picked to a separate plate. A single suppressor of genotype *dpy-2 wee-1.3(e1947;sup)/dpy-2 unc-4* was identified as a self-fertile hermaphrodite that segregated Dpy and DpyUnc progeny. This candidate was crossed to *dpy-2 unc-4/mnC1* males. Non-Dpy outcross progeny were picked to plates and a balanced line of putative genotype *dpy-2 wee-1.3(e1947;sup)/mnC1* was established from hermaphrodites that failed to segregate DpyUnc progeny. No extragenic suppressors were identified in either the γ -ray or ENU screen.

smg suppression

Class 2 *wee-1.3* suppressors (*q89 eb60*, *q89 eb93* and *q89 eb87*) were tested for *smg* suppression. *wee-1.3(q89 eb60) unc-4/mIn1*, *wee-1.3(q89 eb93) unc-4/mIn1* or *wee-1.3(q89 eb87) unc-4/mIn1* males were crossed to *dpy-1 smg-6* hermaphrodites. NonDpy nonGFP F₁ *wee-1.3(q89 sup) unc-4/++*; *dpy-1 smg-6/++* hermaphrodites were picked. F₁ hermaphrodites were allowed to self-fertilize and the DpyUnc *wee-1.3(q89 sup)unc-4*; *dpy-1 smg-6* and Dpy nonUnc *wee-1.3(q89 sup) unc-4/+*; *dpy-1 smg-6* or *+/+*; *dpy-1 smg-6* progeny were picked. For both *q89 eb60* and *q89 eb93*, 100% of the DpyUnc and ~75% of the Dpy non-Unc show a Spe phenotype that is identical to *wee-1.3(q89)/+*. *q89 eb87* was unaffected by the absence of the SMG surveillance system.

Nucleic acid methods

The chromosome II deficiency *ebDf1* was mapped by polymerase chain reaction (PCR)-based methods described previously (Williams, 1995). Each PCR had a control primer pair that would produce a product from the genomic DNA present in *ebDf1* homozygotes and a second pair of test primers. PCR on wild-type embryos was carried out in parallel, and reactions were visualized on ethidium bromide stained agarose gels. At least five PCRs were attempted with each pair of test primers to determine if they could produce a product from *ebDf1* template DNA. This approach revealed that the left *ebDf1* breakpoint is within a 4 kb interval present in cosmid ZK1320 and the right breakpoint is within a 23 kb interval starting in cosmid ZK938 and extending through Y53C12C (data not shown). These data indicate that *ebDf1* deletes ~125-150 kb on chromosome II, including the *C. elegans* Myt1 ortholog, *wee-1.3* (see www.wormbase.org). The *wee-1.3* candidate gene was sequenced from each ENU induced *wee-1.3(q89;sup)* suppressor. When suppressor homozygotes were viable, individual nonGFP *wee-1.3(q89;sup) unc-4* animals were picked from *wee-1.3(q89;sup) unc-4 /mIn1* GFP+ parents and used to prepare template DNA (Williams, 1995). For lethal *wee-1.3* null mutants, nonviable embryos were used to prepare template DNA (Williams, 1995). At least four independent Taq-generated PCR products were fractionated by agarose gel electrophoresis and purified using the GeneClean system (Bio101, Vista, CA). Sequencing was performed at the Iowa State University (Ames, IA) DNA sequencing facility by primer walking using standard ABI automated sequencing. Sequence was analyzed using the DNASTAR software package (DNASTAR, Madison, WI).

To sequence *wee-1.3(gf)* mutants, hermaphrodites of genotype *wee-1.3(gf) unc-4/mIn1* GFP+ were crossed to *dpy-2 ebDf1/mIn1* males.

The nonGFP F1 hermaphrodites [*wee-1.3(gf) unc-4/dpy-2 ebDf1*] were picked to verify the dominant Spe phenotype. Individual animals were prepared for PCR as described for deficiency mapping. Multiple PCR reactions were pooled and sequenced, as described above, to identify the molecular lesions associated with *wee-1.3(gf)* mutants. All mutations were verified by sequencing the wild-type N2 strain (var. Bristol).

A *wee-1.3* rescuing transgene was prepared by high-fidelity PCR (Advantage2, Clontech Laboratories, Palo Alto, CA). A sense primer (TL49 – 5'-ATGTATTAGCATCGTTCTTTAAACCCCAACCAT-3') just outside the predicted 3' end of Y53C12A.1 ORF and an antisense primer (TL55 – 5'-GCAAGAAAATAAAGGAGCGCAAACAAGAGT-3') just outside the predicted 5' end of the Y53C12A.6 were used to amplify a 4.3 kb fragment from N2 genomic DNA. This PCR fragment was microinjected with the dominant *rol-6 (su1006)* encoding plasmid *pRF4* (Mello et al., 1991) into *wee-1.3(q89 eb88) unc-4/mln1GFP+* hermaphrodites. F1 rollers were isolated and a balanced, stable transgenic line was used to assay for *wee-1.3* rescue. This line segregates viable non-GFP Uncs that do not have a germline. The F1 of hermaphrodites microinjected with this and other *wee-1.3*-containing PCR fragments frequently died as embryos (data not shown).

The tissue specificity of the *wee-1.3* promoter was analyzed by ligating it to the green fluorescent protein (GFP)-coding sequence (Chalfie et al., 1994). High-fidelity PCR (Advantage2, Clontech) with primers TL49 (5'-ATGTATTAGCATCGTTCTTTAAACCCCAACCAT-3') and TL50 (5'-CAACTCGAGCATGCCTGCGGAGTGACCAAAAG-3') allowed 1 kb of sequence 5' to the transcriptional start plus the first *wee-1.3* exon to be amplified from N2 genomic DNA. These primers introduce *SphI* (TL49) and *KpnI* (TL50) sites into the resulting PCR product, which was restriction digested with these enzymes. This fragment was ligated into the *SphI/KpnI*-digested GFP-encoding plasmid pPD95.77 (A. Fire, S. Xu, J. Ahnn and G. Seydoux, personal communication) to create pSTL1. pSTL1 (10 ng/μl) was microinjected together with pMH86 (Han and Sternberg, 1991) *dpy-20+* rescuing plasmid (100 ng/μl) into *dpy-20(e1280ts)*. Injected animals were grown at 25°C and transformed animals were identified as non-Dpy F1. Stable lines were used to determine the pattern of GFP fluorescence.

Light and electron microscopy

Light microscopy and immunofluorescence were performed as previously described (Arduengo et al., 1998). Post-acquisition image analysis was performed using ImagePro (Media Cybernetics, Silver Springs, MD) and VolumeScan (Vaytek, Fairfield, IA) software. Electron microscopy was performed as previously described (L'Hernault and Roberts, 1995). Figures were assembled using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA) or Canvas 7 (Deneba Systems).

RESULTS

All dominant *spe* mutants map close to *unc-4* on chromosome II

The *C. elegans* hermaphrodite is somatically female and has a germline that first produces sperm and then switches to produce oocytes. Ovulated eggs are fertilized internally by either the hermaphrodite's own sperm (self-fertility) or sperm inseminated by a male (cross-fertility). This work began as a study of the dominant sterile mutant *e1947/+*, which was isolated in a screen for dominant mutants in the sex determination pathway (Doniach, 1986). Further analysis of *e1947/+* hermaphrodites revealed that this mutant is self-sterile but outcross fertile. As oocytes can be fertilized in

wild type

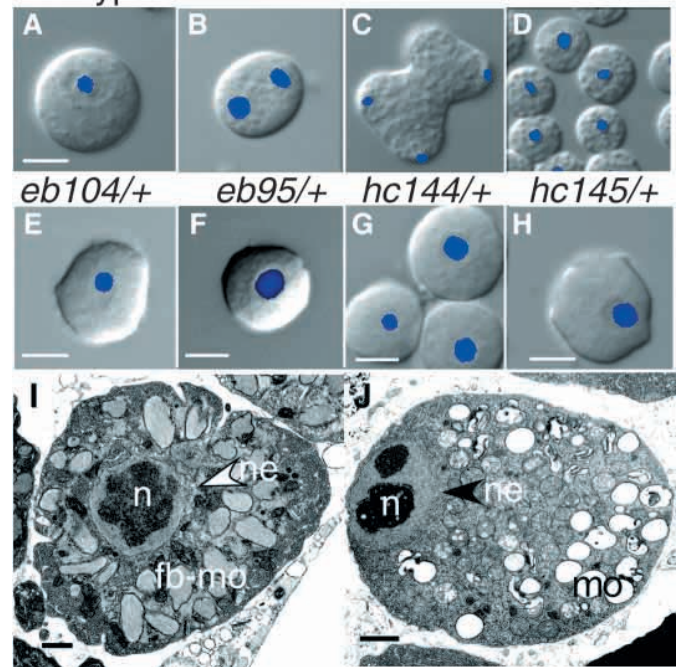


Fig. 1. Light and ultrastructural phenotypes of *spe-37(gf)/+* mutant sperm. (A-D) Various stages of wild-type spermatogenesis at identical magnifications. (A) Primary spermatocyte; (B) dividing primary spermatocyte; (C) dividing secondary spermatocytes; (D) spermatids. (E-H) Terminal phenotype of various *spe-37(gf)/+* mutant spermatocytes. The independently derived *eb1947*, *q89* (not shown) and *e104* mutants bear the identical *spe-37* mutation. (I, J) Transmission EM of *spe-37(e1947)/+* spermatocytes: (I) early mutant phenotype; (J) terminal mutant phenotype. n, nucleus; fb-mo, fibrous body-membranous organelle; mo, membranous organelle; ne, nuclear envelope. Scale bars: 5 μm in A-H; 1 μm in I, J.

e1947/+ hermaphrodites and the mutant displays defective spermatogenesis rather than a sex-determination phenotype, it behaves like a dominant spermatogenesis-defective (*spe*) mutant (L'Hernault, 1997) and was initially named *spe-37(gf)*. Three factor mapping of *spe-37(e1947)* placed it between *dpy-2* and *unc-4* on chromosome II. Five other independently derived dominant *spe* mutants also mapped to chromosome II (*q89*, *eb95*, *eb104*, *hc144* and *hc145*) and four of these were mapped between *dpy-2* and *unc-4* (data not shown). Dominant sterility is not the result of haploinsufficiency because many deficiencies extend through this region and heterozygotes bearing these deficiencies are self-fertile (Shen and Hodgkin, 1988; Sigurdson et al., 1984).

Spermatogenesis in all dominant *spe* mutants arrest at an early stage

Sperm from the dominant *spe* mutants were analyzed by light microscopy. Wild-type spermatocytes progress through meiosis and differentiation in an invariant manner (Fig. 1A-D), with each male producing hundreds of haploid spermatids. By contrast, all dominant *spe* mutant males accumulate arrested primary spermatocytes and no spermatids are observed (Fig. 1E-H). These mutant spermatocytes have a condensed nucleus that is often asymmetrically located, which are characteristic

of later stages of spermatogenesis. Spermatocyte cytokinesis and condensed chromosomes aligned on the metaphase plate have not been observed in any of the dominant *spe* mutants. The same spectrum of cellular defects was observed when a dominant *spe* mutation was in trans to a non-complementing deficiency (data not shown), so this phenotype is neither dependent on the presence of the wild-type allele nor improved by its absence. No dominant *spe* mutant shows obvious evidence of earlier germline defects (data not shown).

Dominant *spe* mutants partially differentiate in the absence of cell division

The defects associated with *spe-37(e1947)/+* and *q89/+* were examined by electron microscopy and both show identical ultrastructural phenotypes. Initially, *spe-37(e1947)/+* (Fig. 1I) and *q89/+* (not shown) appear similar to wild type. Specifically, these mutants contain an uncondensed, centrally located nucleus that is surrounded by a nuclear envelope and normal ER/Golgi-derived fibrous body-membranous organelles (FB-MOs). Terminal *spe-37(e1947)/+* spermatocytes contain condensed chromatin inside an intact nuclear envelope, FBs are not observed and MOs are highly vacuolated (Fig. 1J). These terminal spermatocytes polarize and place their nucleus and FB-MOs on opposite sides of the cell, as if attempting to differentiate. However, this attempt at differentiation occurs without karyokinesis or cytokinesis, suggesting that these dominant *spe* mutations affect a gene that coordinates the meiotic divisions with spermatocyte differentiation.

Spermatocytes in dominant *spe* mutants have phosphorylated histone H3

Although spermatocytes in dominant *spe* mutants do not divide, they do enter pachytene of meiotic prophase I (data not shown). Therefore, we postulated that they are unable to complete M phase. To test this hypothesis, wild-type and *q89/+* sperm were stained with an antibody that recognizes phosphorylated histone H3, a well-characterized marker for M phase/chromosome condensation (Boxem et al., 1999; Golden, 2000; Hsu et al., 2000; Strahl and Allis, 2000). In wild-type, phospho-histone H3 staining is observed in dividing

spermatocytes but not in spermatids (Fig. 2A-D). In *q89/+* males, all spermatocytes contain phospho-histone H3 (Fig. 2E-H). This suggests that dominant *spe* spermatocytes arrest with condensed chromosomes, possibly at the G2/M phase boundary of meiosis I.

Establishing that *spe-37(e1947)/+* and *q89/+* are allelic

Dominant mutants can be subjected to mutagenesis and screened for a new recessive mutation that suppresses the dominance caused by the original mutation (Greenwald and Horvitz, 1980; Conradt and Horvitz, 1998). When the dominant mutation and its recessive suppressor show tight linkage, both mutations frequently reside in the same gene. An allelic series of such second site intragenic suppressor mutations often reveals the null phenotype of that gene. Using this technique, nine ethylnitrosourea (ENU) induced suppressors of *q89* and one γ -ray-induced suppressor of *spe-37(e1947)* were recovered. All suppressor mutations genetically map very close to *q89* and could be unambiguously placed into one of three distinct phenotypic classes (Table 1). Each suppressor mutation functions in cis to *q89*, suggesting that suppression is intramolecular in nature. Furthermore, suppressors derived from *q89/+* fail to complement the suppressor derived from *spe-37(e1947)/+*, indicating that *q89* and *e1947* are both *spe-37* alleles.

spe-37(q89)/+ G2/M meiotic arrest is partially alleviated by Class 1 suppressors

The class 1 suppressor mutant *spe-37(q89 eb61)/+*, *spe-37(q89 eb62)/+* and *spe-37(q89 eb94)/+* hermaphrodites, unlike *spe-37(q89)/+*, are self-fertile. Class 1/+ hermaphrodites have brood sizes that are significantly smaller than those produced by wild type, and they also produce an unusually high percentage of males (Table 1). *C. elegans* males are XO and usually arise in a wild-type hermaphrodite population by X chromosome nondisjunction at a rate of ~0.1% (Brenner, 1974). The higher frequency of males suggests that an increased rate of X chromosome nondisjunction could be occurring in Class 1/+ hermaphrodites. Alternatively, Class 1/+ suppressor mutants might produce a high incidence of males because some gametes lose the X chromosome by another type of meiotic aberration. All three Class 1 suppressor homozygotes, in addition to *spe-37(q89 eb62)/Df* and *spe-37(q89 eb94)/Df* hemizygous hermaphrodites are self-sterile, laying unfertilized oocytes and inviable embryos. Given the increased frequency of X chromosome segregation abnormalities, autosomal segregation might also be aberrant and thus contribute to the observed embryonic lethality. Unlike self-sterile *spe-37(q89 eb61)* homozygous hermaphrodites, *spe-37(q89 eb61)/Df* hemizygotes produce a few viable progeny (mean=11 progeny; $n=63$); therefore two copies of *spe-37(q89 eb61)* results in a more severe phenotype than one copy. All Class 1 heterozygous, hemizygous and homozygous hermaphrodites produce large numbers of cross progeny after mating to wild-type males. Thus, Class 1 homozygotes and hemizygotes have defective sperm but are largely or completely unaffected in somatic development or other aspects of germline development (Table 1).

While *spe-37(gf)/+* males never complete spermatogenesis (Fig. 1E-H), the modest self-fertility shown by Class 1/+

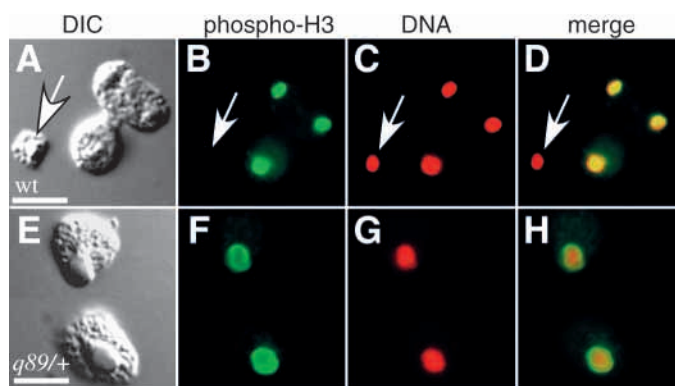


Fig. 2. Phospho-histone H3 staining of wild-type and *spe-37(gf)/+* sperm. (A-D) Wild-type. (E-H) *spe-37(q89)/+*. (A,E) DIC; (B,F) FITC anti-phosphorylated histone H3; (C,G) DAPI staining of nuclei; (D,H) merge of DAPI and anti-phospho-H3. Arrow indicates a wild-type spermatid that does not stain for phospho-histone H3. Scale bars: 5 μ m.

Table 1. Phenotypes of *spe-37(q89)/+* *cis* suppressor mutants

| Class* | Allele | Brood size [†] (sup/+) | % males [‡] | Homozygous phenotype [§] | Molecular lesion [¶] | |
|--------|--------------------------------------|------------------------------------|------------------------|---|-------------------------------|------------|
| | | | | | Nucleotide | Amino acid |
| | Wild type <i>spe-37(q89)</i> | 300±50** 0 | 0.1 ^{††} 0 | Dominant Spe | G1957A | G560R |
| 1 | <i>spe-37(q89 eb62)</i> | 5±6 | 5.7 | Viable, lays oocytes, cross-fertile after mating to wild-type males. Mutant males contain abnormal sperm. | T550A | I160N |
| 1 | <i>spe-37(q89 eb94)</i> | 72±33 | 3.4 | | T378A | F103I |
| 1 | <i>spe-37(q89 eb61)</i> | 95±43 | 3.6 | | G1233A | splicing |
| 2 | <i>spe-37(q89 eb60)</i> | 244±31 | 0.4 | Viable, lays inviable embryos; lays viable embryos if mated to wild-type males. Mutant males are fertile | T2048A | L590stop |
| 2 | <i>spe-37(q89 eb93)</i> | 249±31 | 0.3 | | C2041T | Q588stop |
| 2 | <i>spe-37(q89 eb87)^{‡‡}</i> | 225±33 | 0.3 | | A1279G | splicing |
| 3 | <i>spe-37(q89 eb88)</i> | 248±26 | 0.3 | Inviably embryos and larvae. | G805A | G245E |
| 3 | <i>spe-37(q89 eb90)</i> | 253±16 | 0.3 | | A559C | H163P |
| 3 | <i>spe-37(q89 eb91)</i> | 255±20 | 0.1 | | Δ-80 to 465 | Δ1 to 131 |

*Class was assigned based on the level of self-fertility and the homozygous phenotype of the suppressor mutant.

[†]Except for wild type, brood sizes are the number of self-progeny±s.e.m. produced by heterozygous hermaphrodites grown at 20°C. All suppressor mutations were balanced over either *mnC1* or *mc6*. Progeny counts included animals that were homozygous for the balancer chromosome.

[‡]The percentage of the brood size[†] that are male.

[§]The described homozygous suppressor mutant phenotype is that shown by animals derived from balanced heterozygous hermaphrodites, which means that the wild-type maternal component was present during their development.

[¶]Nucleotide sequence is that found in the gene. '1' corresponds to the 'A' of the initiation 'AUG' codon or the start methionine.

**Singson et al., 1998.

^{††}Brenner, 1974.

^{‡‡}The Class 2 mutant phenotypes are variable. *eb60* and *eb93* suppression of *q89* is slightly temperature sensitive, and heterozygous hermaphrodites lay a mixture of oocytes and viable embryos at 25°C. *eb87* is placed in Class 2 based on the completeness of its *q89* suppression. However, *eb87/eb87* hermaphrodites lay oocytes instead of inviable embryos. Some of these oocytes can be fertilized by wild-type sperm, after which they develop normally.

suppressor hermaphrodites suggests that spermatogenesis is occasionally completed in these animals (Table 1). Class 1/+ mutant males contain many spermatid-like cells. Therefore, some spermatocytes in Class 1/+ suppressor mutants complete the meiotic divisions, something that never occurs in *spe-37(q89)/+* mutants. However, Class 1/+ mutant testes contain many cells that show features never observed during wild-type spermatogenesis, such as 2-4 nuclei within the same cell (Fig. 3). Homozygous Class 1 mutant males contain very few sperm, but those that are present have a phenotype similar to the sperm observed in heterozygous Class 1/+ mutant males (data not shown). Overall, these data indicate that Class 1 suppressor mutants partially alleviate the G2/M meiotic block shown by *spe-37(q89)/+* mutants. Perhaps Class 1 mutants reduce, but do not eliminate, the gain-of-function effects of *spe-37(q89)* and this explains why hermaphrodite brood size and male spermatogenesis are both different from wild type.

Maternal and zygotic functions of *spe-37* are revealed by hypomorphic suppressors

The Class 2 heterozygous *spe-37(q89 eb60)/+*, *spe-37(q89 eb93)/+* and *spe-37(q89 eb87)/+* mutants completely suppress *spe-37(q89)/+* and such suppressed hermaphrodites exhibit wild-type self-fertility (Table 1). Like Class 1 suppressors, homozygous *spe-37(q89 eb60)* and *spe-37(q89 eb93)* Class 2 suppressors are self-sterile, but they produce many more inviable embryos than Class 1 suppressors. Homozygous *spe-37(q89 eb87)* hermaphrodites are self-sterile and lay many unfertilized oocytes but do not produce inviable embryos. All Class 2 mutant hermaphrodites produce large numbers of normal progeny after they are mated to wild-type males. These results suggest that Class 2 alleles are not competent to support

embryonic development and at least one wild-type copy of *spe-37* is required for a zygote to develop normally.

The Class 2 suppressor mutant phenotype is partly dependent on the maternal genotype. When Class 2/+ heterozygous hermaphrodites are crossed to deficiency(Df)/+ males (where Df removes the *spe-37* gene), the resulting Class 2/Df hemizygous hermaphrodites mature into adults that lay a

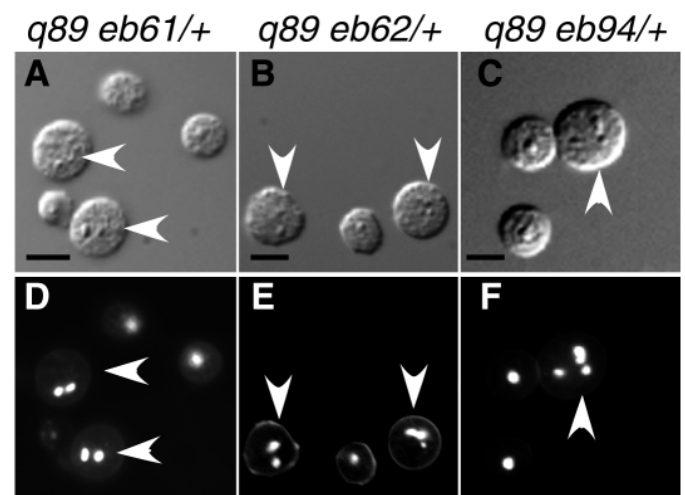


Fig. 3. Light microscopic phenotypes of *spe-37(q89)* Class 1 semi-dominant suppressors. (A-C) DIC and (D-F) DAPI fluorescence light microscopy. (A,D) Sperm from *spe-37(q89 eb61)/+* males; (B,E) *spe-37(q89 eb62)/+* males; (C,F) *spe-37(q89 eb94)/+* males. Arrowheads indicate abnormal sperm with multiple nuclei. Scale bars: 5 μm.

mixture of oocytes and inviable embryos, which is like Class 2/Class 2 homozygous hermaphrodites. However, crossing sterile homozygous Class 2/Class 2 hermaphrodites to Df/+ males, results in inviable Class 2/Df hemizygous embryos that show no morphogenesis. These data suggest that a Class 2/+ mother can maternally supply enough wild-type *spe-37* activity to allow her homozygous Class 2/Class 2 progeny to complete development and begin germline formation. This germline does not function normally, presumably because there is insufficient maternal endowment. When little maternal endowment occurs (Class 2/Class 2 hermaphrodites) and sperm provide no wild-type *spe-37* gene, Class 2 alleles are not able to support embryonic development. These phenotypic properties are not as severe as Class 3 suppressor mutants (see below), which suggests that Class 2 suppressor mutations are loss-of-function but non-null (hypomorphic) alleles.

The many inviable embryos produced by Class 2/Class 2 homozygous hermaphrodites suggests that Class 2 mutants contain spermatozoa that are competent for fertilization. Microscopic examination of dissected heterozygous Class 2/+ or homozygous Class 2/Class 2 mutant males revealed that they contained cytologically wild-type spermatids (data not shown). These spermatids can form functional spermatozoa because hemizygous Class 2/Df mutant males sire viable progeny when crossed to wild-type hermaphrodites. As Class 2 mutants are hypomorphic, these data suggest that reduced *spe-37* gene activity still allows apparently normal spermatogenesis.

Two Class 2 suppressor mutants (*eb60* and *eb93*) are affected when the SMG surveillance system is genetically disabled (see Materials and Methods). The wild-type SMG surveillance system degrades mRNAs that are defective, and a loss-of-function mutation in any of seven *smg* genes eliminates this ability to detect and degrade defective mRNAs (Mango, 2001). Heterozygous *spe-37(q89 eb60)/+* or *spe-37(q89 eb93)/+*, which are fertile in a wild-type SMG background, become dominant Spe when the SMG surveillance system is genetically disabled. Similarly, homozygous *spe-37(q89 eb60)* or *spe-37(q89 eb93)*, which produce inviable embryos in a wild-type SMG background, become dominant Spe when the SMG surveillance system is genetically disabled. In both cases, the observed dominant Spe phenotype is highly similar to *spe-37(q89)/+*. These results suggest that *spe-37(q89)*-associated dominance is suppressed because *eb60* or *eb93* can, respectively, trigger *spe-37(q89 eb60)*- or *spe-37(q89 eb93)*-encoded mRNA degradation by the wild-type SMG surveillance system. Manipulation of the SMG surveillance system in the Class 2 suppressor double mutant background also provided a way to create what are effectively *spe-37(q89)* homozygotes, and *spe-37(q89)/+*, *spe-37(q89)/spe-37(q89)* and *spe-37(q89)/Df* (using deficiencies that remove the *spe-37* gene) all exhibit the same phenotype. These data suggest that the *spe-37(q89)* mutation is insensitive to gene dose and thus may be constitutively active or neomorphic gain-of-function in nature [activity at an inappropriate place or time (Muller, 1932)].

The null phenotype of *spe-37* is embryonic and larval lethal

Like Class 2 mutants, Class 3 *spe-37(q89 eb88)/+*, *spe-37(q89 eb90)/+* and *spe-37(q89 eb91)/+* mutant heterozygous hermaphrodites all exhibit wild-type self-fertility (Table 1). Class 3 homozygotes derived from a Class 3/+ mother receive

a maternal endowment of SPE-37 but, unlike Class 1 and 2 mutants, Class 3 homozygous mutants always die. Usually, Class 3 homozygous mutant embryos die after limited morphogenesis but, occasionally, they hatch into abnormal larvae that die. These data suggest that in wild-type, there is limited maternal SPE-37(+) and that the zygotic embryonic lethality of Class 3 homozygotes is the result of Class 3 alleles that provide little or no functional SPE-37 activity.

Among individual Class 3 mutants, *spe-37(q89 eb91)* causes the least severe phenotype because it produces fewer inviable embryos and more inviable L1 or L2 progeny than do either *spe-37(q89 eb88)* or *spe-37(q89 eb90)* (data not shown). The *spe-37(q89 eb88)/Df* or *spe-37(q89 eb90)/Df* (where the *Df* removes the *spe-37* gene) -associated phenotypes are identical to that shown by the respective mutant homozygotes. This suggests that these Class 3 double mutants exhibit the *spe-37* null phenotype because lowering the gene dose has no effect on the observed phenotype.

A candidate gene approach reveals that *spe-37* is *wee-1.3*

The *spe-37(e1947)* γ -ray induced suppressor failed to complement the chromosome II deficiencies *mnDf63*, *mnDf58*, *mnDf29*, *mnDf57* and *eDf21* with regard to embryonic lethality and its phenotype is similar to the Class 3 *spe-37(q89 eb88)* or *spe-37(q89 eb90)* suppressor double mutants. The *spe-37(e1947)* γ -ray-induced suppressor also failed to complement *mab-3* and all ENU-induced *spe-37(q89)* suppressors, so it behaved genetically like a deficiency and was named *ebDf1*. As *ebDf1* mapped to the same location as *spe-37(e1947)*, we hypothesized that it deleted the *spe-37* gene and other adjacent genes. This hypothesis proved correct and the breakpoints of *ebDf1* define a region that includes the *spe-37* gene.

Although there are at least 26 predicted genes in the ~125-150 kb interval deleted by *ebDf1*, the only obvious cell cycle regulatory gene in this region is *wee-1.3* (see www.wormbase.org). As the *spe-37* associated phenotype suggests it encodes a cell cycle regulatory protein, we hypothesized that *spe-37* and *wee-1.3* were the same gene. This hypothesis was confirmed by showing that transgenes containing the wild-type *wee-1.3* genomic sequence could rescue Class 3 *spe-37(q89 eb88)* homozygotes from embryonic lethality. Although viable, rescued transgenic Class 3 mutants are sterile because they do not form a germline. Prior work has shown that *C. elegans* transgenes frequently express in somatic tissues but show no germline expression because of epigenetic silencing (Kelly and Fire, 1998; Kelly et al., 1997). Although inconvenient, this co-suppression phenomenon permits an assessment of whether a gene must be expressed in the germline for normal germline development. The co-suppression phenotype suggests that *wee-1.3* expression is required for establishment and/or proliferation of the germline.

Each of the six dominant *spe-37* mutants proved to have a point mutation in the *wee-1.3* gene (Fig. 4). Three of these dominant alleles (*q89*, *e1947* and *eb104*) contained the same G1957A mutation that changes the encoded glycine at position 560 to an arginine. *hc144* also affects glycine 560 but this mutation changes it so that glutamate (nucleotide change: G1958A) is encoded. *eb95* converts the encoded glycine at position 558 to an arginine (nucleotide change: G1951A) and *hc145* converts the encoded aspartic acid at position 561 to an

asparagine (nucleotide change, G1960A). Remarkably, all six *spe-37(gf)* mutations affect a four amino acid region in the C-terminal region of *wee-1.3*.

Each of the nine ENU induced *spe-37(q89)* suppressor double mutants had two mutations in *wee-1.3* (Fig. 4). Each suppressor double mutant had the G1957A point mutation (see Table 1) present in the parental *spe-37(q89)* mutant. The second mutation was unique to each suppressor mutant (Fig. 4; Table 1). These DNA sequencing results and *wee-1.3* transgenic rescue unambiguously show that *spe-37* is the *C. elegans* Myt1 ortholog *wee-1.3* (Wilson et al., 1999).

spe-37 suppressor mutations affect key residues in this Wee1-like kinase and an alignment of WEE-1.3 (=SPE-37) to its orthologs helps in interpreting some of the suppressor mutations (Fig. 5). The Class 1 suppressors include two missense mutations (*eb62* and *eb94*) and one mutation (*eb61*) in a splice donor site (Fig. 4; Table 1). The *eb62* suppressor mutation is the weakest Class 1 suppressor and it allows hermaphrodites to produce broods of approx. five progeny (Table 1). *eb62* is an I160N missense mutation in an amino acid that shows weak conservation between *wee-1.3* and its vertebrate orthologs (Fig. 5). The *eb94* suppressor mutation allows hermaphrodites to produce broods of ~72. The *eb94* F103I missense mutation affects a residue that is conserved among Myt1 orthologs (Fig. 5) but not among other members of the Wee1p kinase family (Wilson et al., 1999). The *eb61* mutant permits the largest brood (~95; Table 1) of any of the Class 1 suppressors, and it converts the fourth intron splice donor into an in-frame codon (Fig. 4 and Table 1). The Genemark HMM program (Borodovsky, 1998) suggests that other upstream consensus splice donor sites are present (data not shown), and the incompleteness of suppression suggests that such sites might be used.

The Class 2 suppressors include two premature stop mutations (*eb60* and *eb93*) and one splice acceptor mutation in intron 4 (*eb87*; Fig. 4A). The molecular natures of *eb60* and *eb93* are consistent with their ability to be suppressed by mutants in the SMG mRNA surveillance system, as some *smg*-suppressible mutations are premature stop codons (Mango, 2001). Both *eb60* and *eb93* cause UAA ochre stop codons that would truncate the polypeptide sequence near the C terminus. The *eb87* mutation alters a conserved splice acceptor site and RT-PCR of mutant animals shows that exon 5, which contains the transmembrane domain for WEE-1.3, is skipped (Fig. 4B). Additionally, the mechanism of *eb87* suppression is not through a SMG-mediated reduction in mRNA levels, as *eb87* is unchanged in a *smg* mutant background.

The Class 3 suppressors include two missense mutations (*eb88* and *eb90*) and one small deletion (*eb91*) (Fig. 4; Table 1). The *eb88* suppressor results in a G245E missense mutation and *eb90* results in a H163P missense mutation; each of these mutations affects an amino acid that is within a strongly conserved region (Fig. 5) found in all Wee1p kinases from yeast to humans (Wilson et al., 1999). The *eb91* suppressor mutation is a 545 bp deletion that removes the first 465 bp of coding sequence and 80 bp 5' to the start codon. It is unlikely that *wee-1.3* is transcribed in *eb91* suppressor mutants because part of the promoter and the entire 5' untranslated region, including the

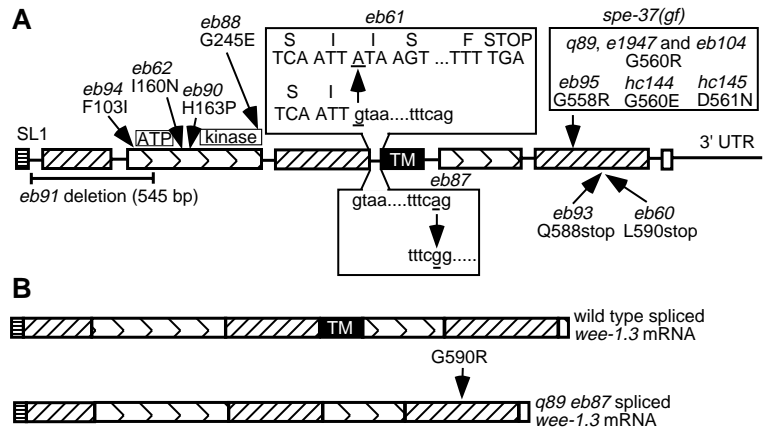


Fig. 4. Genomic structure of *C. elegans wee-1.3* and location of mutations. (A) The eight exons (including the *trans* spliced SL1 leader exon) of *wee-1.3* RNA are indicated by differently shaded or filled rectangles. Introns are the lines between exons. The *spe-37(gf)* mutations are listed within a large box above exon 7. The missense suppressor mutations (*eb94*, *eb62*, *eb90* and *eb88*) and the associated amino acid changes are indicated above exon 3. The two suppressor mutations that cause a premature stop (*eb93* and *eb60*) are indicated below exon 7. The *eb91* deletion that removes exon 2 and part of exon 3 (including the intron acceptor for *trans* spliced exon 1) is indicated below these exons. The *eb61* suppressor mutation alters the intron 4 splice donor (shown in the box above intron 4), while the *eb87* suppressor alters the intron 4 splice acceptor site mutation (shown in the box below intron 4). (B) Comparison of *wee-1.3* mRNA produced by wild-type (top) and *spe-37(q89 eb87)* suppressor mutant homozygotes (bottom). Splicing is altered so that the exon 5 sequence encoding the predicted transmembrane domain is absent.

intron *trans*-splice acceptor for SL1 (A. Golden, personal communication), are missing. Consequently, its molecular features indicate that *eb91* is a *wee-1.3* null mutation.

***wee-1.3* is widely expressed during *C. elegans* development**

The recessive lethality shown by the Class 3 *spe-37(q89)* suppressors indicates that transcription of this gene is required outside the testes. Northern hybridization experiments reveal that the *wee-1.3*-encoded 2.4 kb mRNA was found in *fem-1(hc17lf)* hermaphrodites, which do not make sperm, as well as *fem-3(q23gf)* hermaphrodites, which make sperm but no oocytes (data not shown). Tissue specificity of *wee-1.3* was further examined by fusing the promoter and first exon to the coding sequence for GFP and using this construct to create transgenic worms. Seven stable transgenic lines all consistently showed GFP during early embryonic development (Fig. 6A,B), in the distal region of the larval, but not adult, germline (Fig. 6C,D), and in some larval neurons and hypodermal cells (Fig. 6E,F). These data confirm that the *wee-1.3* promoter is active during both germline and embryonic development.

DISCUSSION

***wee-1.3(gf)* has allowed a genetic analysis of the Myt1 kinase family**

We have analyzed a series of six dominant mutations in *wee-1.3* that affect the G/M transition during spermatogenesis but do not affect either the somatic cell cycle, germline

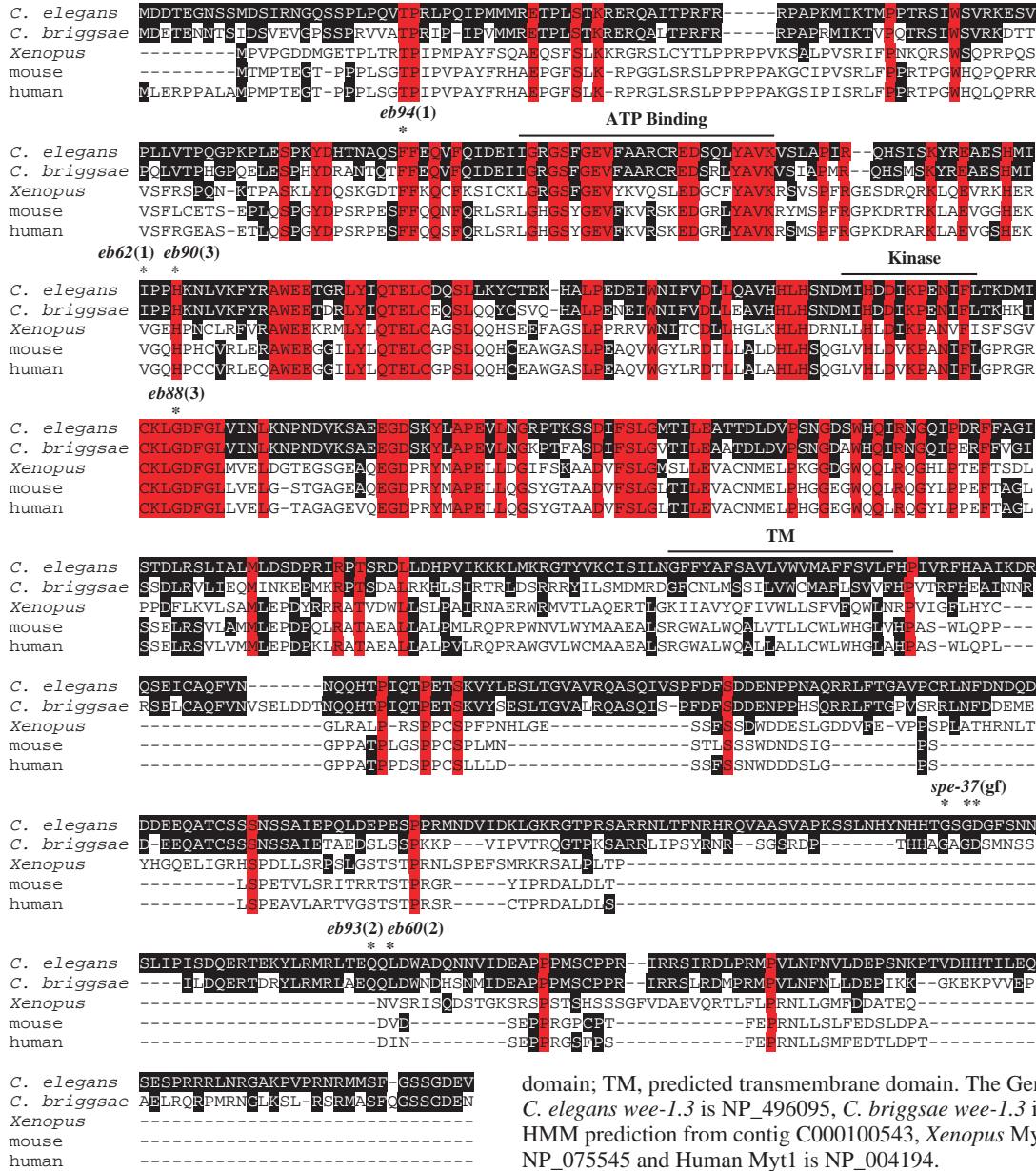


Fig. 5. Alignment of *C. elegans*, *C. briggsae*, *Xenopus*, mouse and human *wee-1.3* kinases. Red boxes indicate identical residues among all *wee-1.3* orthologs. Black boxes indicate identity to the *C. elegans* sequence. Asterisks indicate the sites of mutation in *C. elegans* *spe-37(gf)*, missense and nonsense suppressors. The phenotypic class of each missense or nonsense suppressor is indicated in parentheses (see Table 1). Alignment was performed using the ClustalX program (Thompson et al., 1997). The *C. briggsae* *wee-1.3* sequence is publicly available unpublished data from the *C. briggsae* Genome Project (The Sanger Institute Cambridge, UK and The Genome Sequencing Center, Washington University, St Louis, MO). *C. briggsae* *wee-1.3* cDNA was predicted using GeneMark hmm (Borodovsky, 1998) and sequence around the *spe-37(gf)* region was verified by examination of ABI sequence traces (see <http://trace.ensembl.org>). ATP, ATP-binding domain; kinase, Wee1p-like kinase

proliferation or oogenesis. Each of these dominant mutations affects a residue within a four amino acid region, suggesting that this region is important for regulation of WEE-1.3 during spermatogenesis. Alleviation of the dominant *Spe* self-sterile phenotype allowed isolation of 10 self-fertile intragenic *wee-1.3(gf)* suppressors. These suppressor mutations all reduce WEE-1.3 activity, indicating that the dominant mutants have excess WEE-1.3 activity during spermatogenesis. The suppressor mutations also reveal that *wee-1.3* is an essential gene, required during embryogenesis and for germline proliferation.

Previous *wee-1.3* RNA-interference studies (A. Golden, personal communication) and our work both indicate that WEE-1.3 plays a crucial role during the cell cycle in multiple *C. elegans* cell types. Wee1p kinases are thought to function primarily through their phosphorylation of Cdc2p. This suggests that the dominant spermatogenesis arrest (including

the associated lack of nuclear envelope breakdown) seen in *wee-1.3(gf)* mutants occurs via Cdc2p-negative regulation because active Cdc2p is required for both mitotic and meiotic G2/M progression in all studied eukaryotes (Nurse, 2000). A problem with this interpretation is that *wee-1.3(gf)* mutant spermatocytes have phosphorylated histone H3, which is a known marker for active Cdc2p in the *C. elegans* mitotic germline (Boxem et al., 1999). Phosphorylation of histone H3 is mediated by the Ip11/aurora kinase (Hsu et al., 2000), and activation of this kinase is correlated with the presence of activated Cdc2p (Boxem et al., 1999). Perhaps Ip11/aurora kinase requires a lower level of activated Cdc2p than that needed for nuclear envelope breakdown and entry into M phase.

The transmembrane region found in all Myt1 orthologs separates the conserved, N-terminal kinase domain from a non-conserved C-terminal region (Fig. 4). Overexpression of the C-

domain; TM, predicted transmembrane domain. The GenBank Accession Number for *C. elegans* *wee-1.3* is NP_496095, *C. briggsae* *wee-1.3* is based on the GeneMark HMM prediction from contig C000100543, *Xenopus* Myt1 is A57247, Mouse Myt1 is NP_075545 and Human Myt1 is NP_004194.

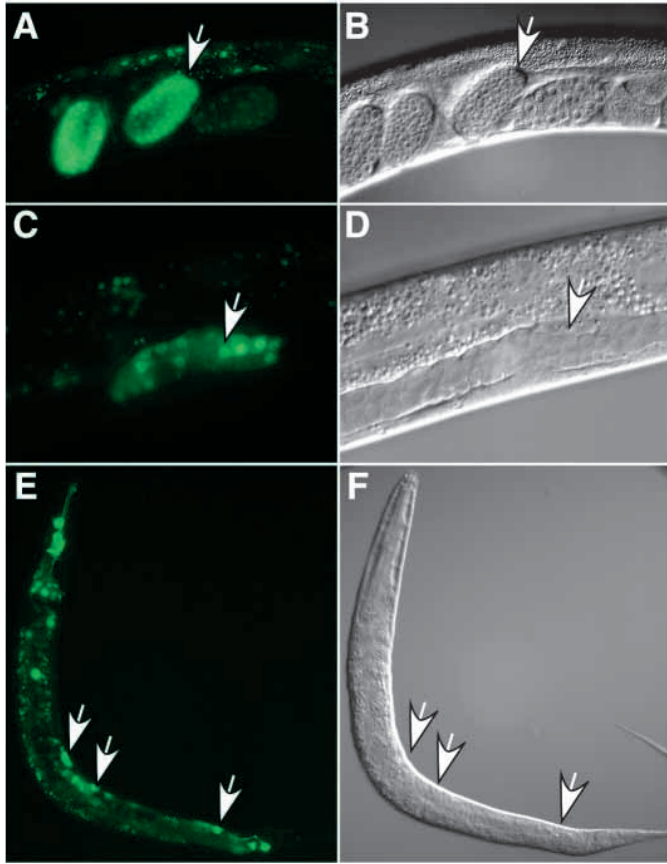


Fig. 6. Expression from a *wee-1.3* promoter-GFP fusion. Paired GFP fluorescent (A,C,E) and DIC (B,D,F) images. Arrowheads indicate (A,B) sites of GFP expression during early embryogenesis; (C,D) larval distal germline proliferation; (E,F) larval neuronal and hypodermal cells. No fluorescence was observed in adult somatic tissues (not shown).

terminal domain in tissue culture cells causes a G2/M phase delay that appears to be mediated by Cdc2p/cyclin (Liu et al., 1999; Wells et al., 1999). The six *C. elegans* *wee-1.3* dominant missense mutations described here affect a four residue motif in the C-terminal region that is not conserved between nematodes and vertebrates at the primary sequence level, but is conserved between *C. elegans* and the closely related *Caenorhabditis briggsae* (Fig. 5). Interestingly, three conserved amino acids within the *wee-1.3(gf)* domain include the residues affected by each of the six *spe-37(gf)* mutations.

Suppressors of the *wee-1.3(gf)* mutant phenotype provide some insight into the role of the Myt1 transmembrane domain. The *wee-1.3(q89 eb87)* suppressor double mutation encodes a WEE-1.3 protein that lacks the transmembrane domain but maintains the dominant missense mutation caused by *wee-1.3(q89)*. The resulting suppression observed in *wee-1.3(q89 eb87)/+* heterozygotes restore wild-type self-fertility (Table 1). Homozygous *wee-1.3(q89 eb87)* mutants survive embryogenesis and grow into self-sterile Spe adults that can produce outcross progeny after mating to wild-type males. This indicates that transmembrane localization of WEE-1.3 is required for the dominant Spe phenotype, but not required for the somatic and oocyte functions performed by this kinase.

The self-sterile phenotype exhibited by *wee-1.3(q89 eb87)* homozygotes could indicate that membrane localization is required for WEE-1.3 to function during spermatogenesis. Alternatively, perhaps the *wee-1.3(q89)* dominant mutation can still affect spermatogenesis in homozygous *wee-1.3(q89 eb87)* suppressor mutants, but only when it does not have to compete with wild-type WEE-1.3.

Mechanism of *wee-1.3(gf)* dominance

The six *wee-1.3(gf)* mutations described in this paper specifically affect spermatogenesis and do not require a wild-type copy of *wee-1.3* to have their effect. *wee-1.3(gf)/Df* animals only produce the dominant mutant form of WEE-1.3 and exhibit the same phenotype as *wee-1.3(gf)/+* animals. As the *wee-1.3(gf)* mutant phenotype is not different when *wee-1.3* gene dose is reduced, the dominance is probably neomorphic/gain of function (gene activity in an inappropriate time or place) in nature. Furthermore, these data indicate that WEE-1.3(gf) can substitute for wild-type during embryonic and oocyte development, but not during spermatogenesis. The WEE-1.3 protein appears to be negatively regulated during spermatogenesis because strong hypomorphic suppressor double mutants, like Class 2 *wee-1.3(q89 eb60)*, mimic negative regulation and restore spermatogenesis in both heterozygous and homozygous mutant animals. The *wee-1.3(gf)* phenotype is tissue specific, and perhaps there is a negative regulator expressed only during spermatogenesis that specifically regulates male meiosis.

Gamete-specific cell cycle regulation has been observed in other organisms and two cases are especially relevant to our study. The *Drosophila twine(lf)* mutant fails to complete the G2/M transition during spermatogenesis but still differentiates, which is similar to spermatogenesis in *wee-1.3(gf)* mutants (Alphey et al., 1992). *Twine* encodes a Cdc25p phosphatase, so *cdc25(lf)* and *wee-1.3(gf)* mutants would both shift cyclinB1/Cdc2p phosphorylation towards the inhibitory state. Deletion of the mouse *Cdc25b* gene has no somatic effects but results in female sterility because oocytes cannot exit meiosis I prophase arrest (Lincoln et al., 2002). These data suggest that metazoan regulation of the gamete cell cycle is fundamentally different from regulation of the somatic cell cycle, and that studies of WEE-1.3 function in *C. elegans* are likely to be applicable to Myt1 function in higher vertebrates.

We thank Jonathan Hodgkin, Jacob Varkey, Tim Schedl and Andy Fire for providing *C. elegans* strains or DNA clones. We thank Andy Golden for many useful discussions and permission to cite unpublished data. Win Sale, Bill Kelly, Kevin Moses, Andy Golden and Penny Sadler all provided useful comments on the manuscript. The *ebDf1* mutation was recovered by S. W. L. while he was a postdoctoral fellow in the laboratory of Samuel Ward, and the continuous support and encouragement of S. W. is appreciated. We thank Alan Shearn for allowing use of his ¹³⁷Cs γ -ray source and Nancy L'Hernault for assistance with electron microscopy. Preliminary unpublished data from the *C. briggsae* genome sequencing project was provided by The Wellcome Trust Sanger Institute, Cambridge, UK and The Genome Sequencing Center, Washington University, St Louis, MO. The *Caenorhabditis* Genetic Center provided some nematode strains, and it is funded by the NIH National Center for Research Resources (NCRR). This work was supported by the US Public Health Service through Training Grant GM008367 to S. T. L. and RO1 GM40697 to S. W. L.

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