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Report

Tyrosines in the Kinesin-5 Head Domain Are Necessary for Phosphorylation by Wee1 and for Mitotic Spindle Integrity

Kristin Garcia,[1] Jason Stumpff,[1,2] Tod Duncan,[1,3] and Tin Tin Su[1,*]
[1]Department of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, CO 80309-0347, USA

Summary

Mitotic spindle assembly and maintenance relies on kinesin-5 motors that act as bipolar homotetramers to crosslink microtubules [1–5]. Kinesin-5 motors have been the subject of extensive structure-function analysis [5], but the regulation of their activity in the context of mitotic progression remains less well understood [2]. We report here that *Drosophila* kinesin-5 (KLP61F) is regulated by *Drosophila* Wee1 (dWee1). Wee1 tyrosine kinases are known to regulate mitotic entry via inhibitory phosphorylation of Cdk1 [6–10]. Recently, we showed that dWee1 also plays a role in mitotic spindle positioning through γ-tubulin and spindle fidelity through an unknown mechanism [11]. Here, we investigated whether a KLP61F-dWee1 interaction could explain the latter role of dWee1. We found that dWee1 phosphorylates KLP61F in vitro on three tyrosines within the head domain, the catalytic region that mediates movement along microtubules. In vivo, KLP61F with tyrosine→phenylalanine mutations fails to complement a *klp61f* mutant and dominantly induces spindle defects similar to ones seen in *dwee1* mutants. We propose that phosphorylation of the KLP61F catalytic domain by dWee1 is important for the motor's function. This study identifies a second substrate for a Wee1 kinase and provides evidence for phosphoregulation of a kinesin in the head domain.

Results and Discussion

dWee1 and KLP61F Interact at Endogenous Protein Levels

Previously, we identified *Drosophila* kinesin-5 (KLP61F) in mass spectrometric analysis of HA-dWee1-containing protein complexes [11] (data not shown). To confirm that dWee1 and KLP61F interact at physiological levels, we assayed for coimmunoprecipitation of endogenous proteins (Figure 1). The specificity of a previously described anti-KLP61F serum [12] and a newly generated anti-dWee1 antibody were first confirmed with western blots of extracts from respective mutants (see Figure S1 available online). The anti-dWee1 antibody recognized a doublet (Figure S1C), similarly seen for human Wee1 [13]. This doublet was present in immunoprecipitates obtained with the anti-KLP61F serum (Figure 1). Also, the anti-dWee1 antibody immunoprecipitated dWee1 and coprecipitated KLP61F from syncytial embryos (data not shown).

GST-dWee1 Phosphorylates His-KLP61F within the Head Domain

Because dWee1 is a tyrosine kinase, we next investigated whether dWee1 could phosphorylate KLP61F. Incubation of recombinant purified His-KLP61F with GST-dWee1 resulted in phosphorylation (Figure 2B) and recognition of the former by an anti-phosphotyrosine antibody (Figure 2C). Autophosphorylation by dWee1 occurred as expected [14, 15]. Mass spectrometry was used to identify phosphorylated peptides of His-KLP61F after GST-dWee1 kinase assays. We achieved 57% coverage of KLP61F and identified four phosphopeptides containing a single tyrosine (see Supplemental Experimental Procedures). Three of these peptides are in the head domain and contain Y23, Y152, or Y207 as their single tyrosine (Figures S1A–S1C). The fourth is in the BimC box, a conserved region of ~20 amino acids in the tail domain, and contains Y927. To confirm that these regions of KLP61F are important for phosphorylation by dWee1, we generated purified polypeptides containing either the head domain (His-Head^{WT}) or the BimC box (His-BimC; Figure 2A). Both were phosphorylated by GST-dWee1, but only His-Head^{WT} was recognized by an anti-phosphotyrosine antibody after phosphorylation by GST-dWee1 (Figures 2D and 2E). Therefore, we focused our analysis on the head domain.

Tyrosines in the Head Domain Are Required for Phosphorylation by dWee1

Mutation of the putative phosphoacceptor residues Y23, Y152, and Y207 in the head domain to phenylalanines (F) resulted in greatly diminished phosphorylation by dWee1 (3YF mutant; Figures 2F and 2H). It is unlikely that these mutations resulted in protein misfolding: mutant head domain was found to bind ATP in preliminary studies (data not shown), and, more importantly, full-length KLP61F with 3YF mutations shows activity in vivo (described below). The head domain of the *Drosophila* kinesin-1 motor, kinesin heavy chain (GST-KHC), lacks tyrosines at the corresponding residues and was also a poor substrate for dWee1 (Figures S1A–S1C; Figures 2F and 2G). Thus, phosphorylation in the head domain by dWee1 appears specific to KLP61F. Of the three putative phosphoacceptor tyrosines in KLP61F, Y207 is conserved in metazoan kinesin-5 motors (Figure S1C). Alignment of residues flanking Y207 of kinesin-5 motors and Y15 of Cdk1 homologs from *Drosophila*, human, and *Xenopus* reveals several conserved residues (Figure S1D). These might comprise a Wee1 consensus if Y207 serves as a phosphoacceptor for Wee1 in other kinesin-5 motors.

Potential Phosphoacceptor Tyrosines Are Important In Vivo

klp61f^{3} is a well-characterized loss-of-function allele with a transposon insertion in the 5′ untranslated region that greatly reduces protein expression [16, 17] (Figure S1A). Neuroblasts from *klp61f*^{3} homozygous larvae have mitotic spindle defects, chromosome segregation failure, and polyploid nuclei that result in death at the pupal stage [16, 17]. A full-length Myc-KLP61F^{WT} transgene expressed constitutively from the ubiquitin promoter has been shown to rescue the lethality

*Correspondence: tin.su@colorado.edu
[2]Present address: Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195-7290, USA
[3]Present address: Department of Integrative Biology, University of Colorado Denver, Denver, CO 80217-3364 USA

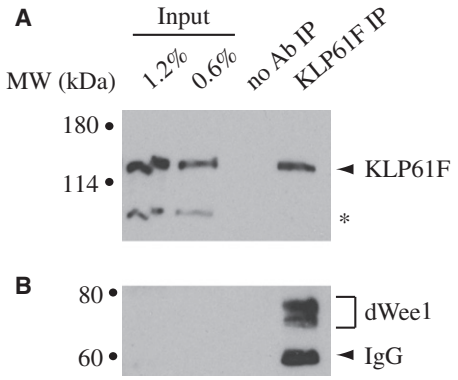

Figure 1. Endogenous KLP61F and dWee1 Interact

Extracts from syncytial wild-type embryos were immunoprecipitated with anti-KLP61F serum or mock precipitated (no Ab IP) and western blotted for KLP61F (A) or dWee1 (B). Samples equivalent to 0.6% and 1.2% of starting extracts were loaded for comparison.

and cytological defects of *klp61f*^{3} homozygous larvae [16]. To determine the importance of Y23, Y152, and Y207 in vivo, we used an identical expression system to express a full-length Myc-KLP61F^{3YF}. Of the five independent transgenic lines generated, we analyzed one that expressed Myc-KLP61F^{3YF} at levels similar to Myc-KLP61F^{WT} (Figure S1A). Unlike Myc-KLP61F^{WT}, Myc-KLP61F^{3YF} did not rescue the lethality of *klp61f*^{3} homozygotes or the incidence of polyploidy in larval neuroblasts (Figures 3A and 3B), suggesting that Myc-KLP61F^{3YF} has reduced function. These data indicate that Y23, Y152, and/or Y207 are important for KLP61F function in larval cells.

Embryos with Reduced *klp61f* Have Spindle Defects

Next, we asked whether Y23, Y152, and Y207 are also important for KLP61F function in embryonic syncytial cycles, where *dwee1* has been implicated in maintaining spindle fidelity [11]. *Drosophila* embryogenesis begins with 13 nuclear divisions driven by maternally contributed products. These divisions occur synchronously in a common cytoplasm called a syncytium and alternate between S and M phases without gap phases. The first nine syncytial divisions occur in the interior of the embryo (interior divisions), after which nuclei migrate outward such that cycles 11–13 occur in a monolayer directly beneath the cortex (cortical divisions). *Drosophila* embryos that lack maternally provided *dwee1* die as syncytial embryos, consistent with other Wee1 homologs being essential for metazoan embryogenesis [18–20].

Embryos from *Drosophila* females hemizygous for the strongest extant allele of *dwee1*, *dwee1*^{ES1} (hereafter referred to as "*dwee1* mutant embryos"), show multiple spindle defects [10, 11]. As expected, these defects included ones known to result from elevated Cdk1 activity and premature mitotic entry. Surprisingly, these defects also included two *dwee1*-specific defects that could not be explained by premature mitotic entry or bulk elevation in Cdk1 activity: multiple microtubule-organizing centers (MTOCs) within a single spindle, and microtubule spurs that extend to neighboring spindles, leading to collisions [11].

To investigate a possible link between dWee1 and KLP61F during syncytial cycles, we wanted to compare mitotic spindles in *dwee1* mutant embryos to mitotic spindles in embryos with reduced KLP61F. Homozygotes of strong *klp61f* mutants do not survive to adulthood [17], but a recent study found that syncytial embryos from *klp61f*^{3} heterozygous mothers exhibit a phenotype of abnormal spindle length [1]. Therefore, we examined these embryos (hereafter referred to as "*klp61f* embryos") for possible additional spindle defects. We found that *klp61f* embryos had reduced KLP61F (Figure S3) and that ~20% of spindles were abnormal (Table 1). Although the incidence of spindle defects was unexpectedly high for embryos that typically survived to adulthood, the majority of these problems occurred during the interior divisions. Defective syncytial nuclei are typically culled into the interior yolk mass and do not contribute to subsequent embryogenesis [21], which might explain the viability of *klp61f* embryos. The most frequent defects were anastral and monopolar spindles (Figures 4B and 4C; quantified in Figure 4D; see Table 1 for a complete listing of all spindle defects). Such defects have been reported in larval neuroblasts from *klp61f* mutants or embryos injected with anti-KLP61F antibodies [17, 22].

Potential Phosphoacceptor Tyrosines Are Important for the Prevention of Monopolar Spindles

Restoring KLP61F with a maternal transgene encoding Myc-KLP61F^{WT} rescued both anastral and monopolar spindles in *klp61f*^{3} embryos ("[WT]" in Figure 4D). To date, it remains unclear what aspect of KLP61F function normally prevents anastral spindles [17]. Nonetheless, anastral spindles in *klp61f*^{3} embryos were similarly reduced when mothers carried a transgene encoding Myc-KLP61F^{3YF} ("[3YF]" in Figure 4D). This suggests that Myc-KLP61F^{3YF} is at least partially active. Consistent with this idea, Myc-KLP61F^{3YF} also localized to mitotic spindles, as did endogenous KLP61F and Myc-KLP61F^{WT} (Figure S3B) [12]. These data, together with the preliminary finding that His-Head^{3YF} can bind ATP in vitro (data not shown), strongly suggest that the 3YF mutations do not create a general protein folding problem for KLP61F.

The Myc-KLP61F^{3YF} transgene, however, did not rescue the monopolar spindle defect in *klp61f*^{3} embryos ("[3YF]" in Figure 4D). The role of KLP61F in the prevention of monopolar spindles has been explained by its ability to antagonize minus-end-directed motors [4, 22]. In the absence of KLP61F, the latter bring separated centrosomes together; this collapses the spindle from a bipolar to a monopolar structure [22]. Consistent with the idea that monopolar spindles in *klp61f*^{3} embryos result from reduced KLP61F, such defects were rescued in [WT];*klp61f*^{3} embryos (Figure 4D). Monopolar spindles, however, were not rescued in [3YF];*klp61f*^{3} embryos (Figure 4D). Thus, potential phosphoacceptor tyrosines in the KLP61F head domain appear to be important for this aspect of KLP61F function.

KLP61F^{3YF} Induces Spindle Defects in a Dominant Manner

The presence of maternal Myc-KLP61F^{3YF} produced two additional spindle defects in *klp61f* embryos. These defects were also seen in *dwee1* mutant embryos and are "*dwee1* specific," i.e., independent of Cdk1 misregulation [11]. One defect was multiple MTOCs per spindle, as defined by staining for centrosomin (Figures 4F and 4G). The incidence of spindles with multiple MTOCs was not significantly different among embryos from wild-type, *klp61f*^{3}/+, and *klp61f*^{3}/+ mothers carrying the Myc-KLP61F^{WT} transgene (wild type, *klp61f*, and [WT], respectively, in Figure 4E). In contrast, embryos from *klp61f*^{3}/+ mothers carrying the Myc-KLP61F^{3YF} transgene displayed a significantly higher incidence of spindles with multiple MTOCs ($p < 0.01$) ([3YF] in Figure 4E; Table 1).

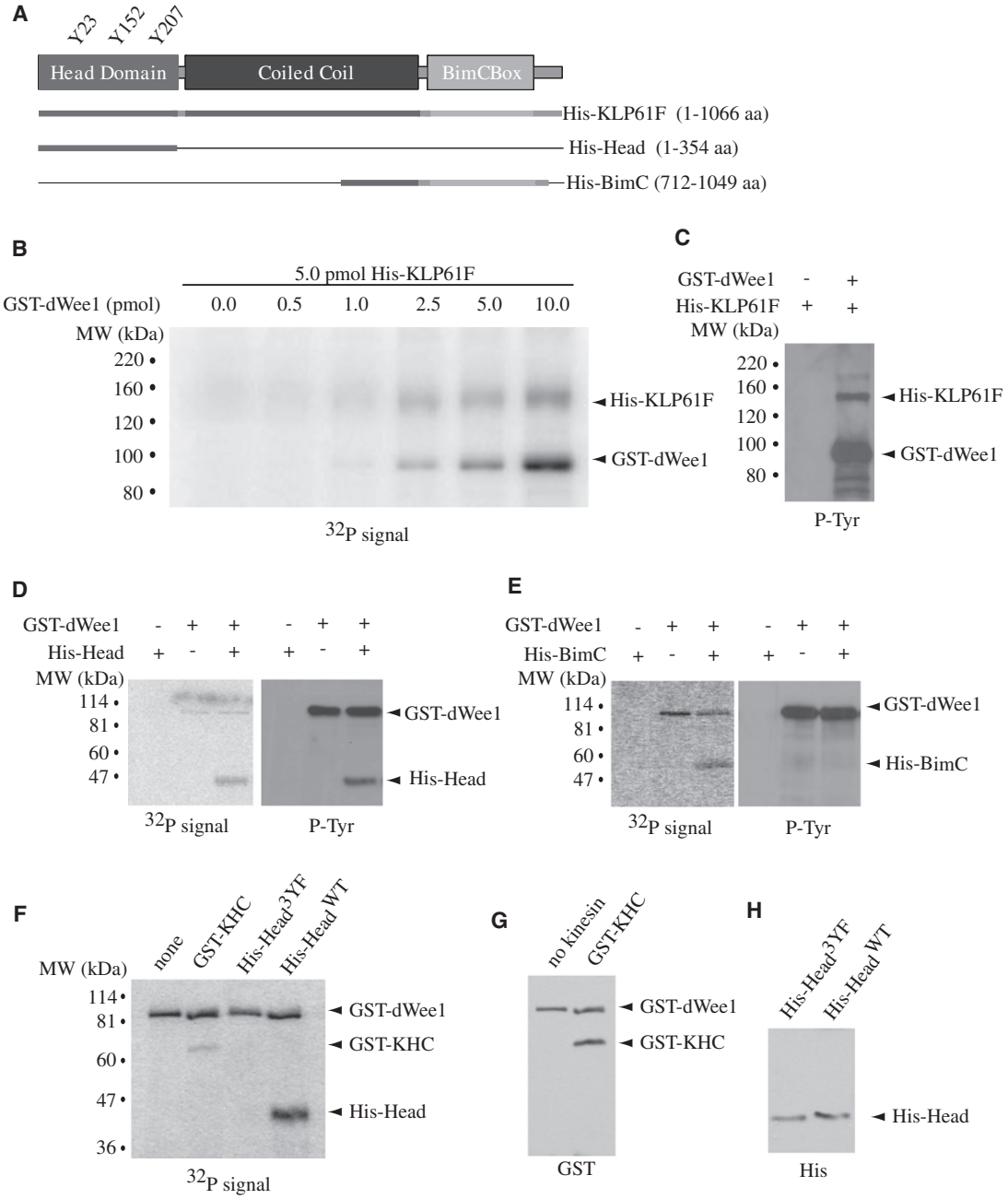

Figure 2. GST-dWee1 Phosphorylates KLP61F on Tyrosines in the Head Domain In Vitro

(A) Schematic representation of KLP61F with head domain, coiled-coil domain, and tail domain containing the BimC box. His-tagged polypeptides used in kinase assays are depicted.

(B) His-KLP61F was incubated with varying amounts of GST-dWee1 in kinase reactions in vitro and analyzed for ^{32}P incorporation.

(C) Reaction with equimolar GST-dWee1 and His-KLP61F was western blotted with anti-phosphotyrosine antibody after in vitro kinase assays.

(D and E) GST-dWee1 and His-KLP61F polypeptides were incubated in in vitro kinase reactions and analyzed for ^{32}P incorporation (left panels) and subsequently by western blots for phosphotyrosines (right panels).

(F) KLP61F head domain with Y23, Y152, and Y207 mutated to phenylalanines, His-Head^{3YF}, was incubated with GST-dWee1 in in vitro kinase reactions and analyzed for ^{32}P incorporation. The following notations are also used: none, no kinesin; GST-KHC, conventional kinesin heavy chain head domain; His-Head^{WT}, wild-type head domain.

(G and H) Western blots for GST (G) or His tags (H) show that equivalent amounts of substrates were used in the kinase reactions shown in (F).

Spindles with multiple MTOCs were also seen in *dwee1* mutant embryos during both interior and cortical divisions ([11] and data not shown).

The second *dwee1*-specific defect induced by Myc-KLP61F^{3YF} in *klp61f*^{3} embryos was promiscuous microtubules, which included microtubule spurs and microtubules interacting between neighboring spindles during cortical divisions (Figure 4H). Because embryo size remains constant, increases in nuclear density lead to crowding of spindles. During cortical divisions, cortical actin descends to form "furrows" separating

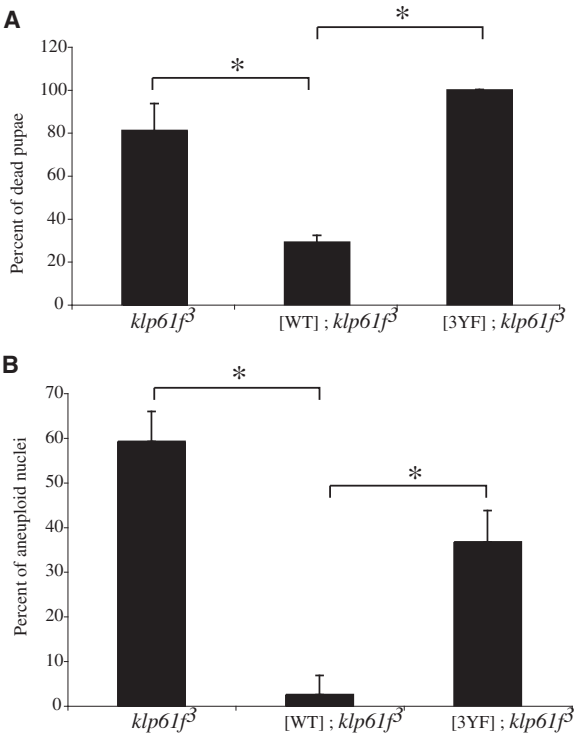

Figure 3. Phosphoacceptor Tyrosines in the Head Domain Are Important In Vivo

Pupal lethality of *klp61f*^{3} mutants (A) and level of aneuploidy in *klp61f*^{3} mutant neuroblasts (B) are rescued by a Myc-KLP61F^{WT} transgene ([WT];*klp61f*^{3}), but not by a Myc-KLP61F^{3YF} transgene ([3YF];*klp61f*^{3}). Data are represented as percentage of dead pupae (mean ± SD from three embryo collections) in (A) and as percentage of aneuploid nuclei (mean ± SD of nuclei from three larval brains) in (B). *p < 0.01.

neighboring spindles. These actin furrows retract during metaphase and are absent in subsequent stages of mitosis, yet spindles do not collide [23]. We had proposed that dWee1 plays a role, via phosphoregulation of γ-tubulin, in positioning nuclei and spindles within the protection of these furrows to prevent spindle collisions [11]. However, in *dwee1* mutant embryos, spindles collide throughout mitosis [11], suggesting that dWee1 acts in another, furrow-independent mechanism that normally prevents spindle interactions after metaphase.

During cortical divisions, we observed promiscuous microtubules in metaphase in *klp61f*^{3} embryos regardless of whether or not the mother carried a transgene; the incidences were not significantly different among *klp61f*^{3}, [WT];*klp61f*^{3}, and [3YF];*klp61f*^{3} embryos (Table 1). However, in anaphase, [3YF];*klp61f*^{3} embryos, but not [WT];*klp61f*^{3} embryos, showed a significant increase in spindles with promiscuous microtubules ($p < 0.05$) (Figure 4E; Table 1). This could be because in [WT];*klp61f*^{3} embryos, regulation by dWee1 ensures optimal activation of transgenic KLP61F and proper spindle integrity (model in Figure 4I). In [3YF];*klp61f*^{3} embryos, the level of transgenic KLP61F was similar to that in [WT];*klp61f*^{3} embryos, but regulation by dWee1 was absent. Furthermore, it appears that having elevated KLP61F that cannot be regulated (in [3YF];*klp61f*^{3} embryos) is worse than having reduced KLP61F that can be regulated, because *klp61f*^{3} embryos do not show anaphase spindle interactions.

Table 1. Percent of Spindle Defects in Syncytial Embryos

	Wild-Type	*klp61f*	[WT]	[3YF]
Interior Divisions (Cycles 1–9)	n = 201	n = 532	n = 287	n = 410
Monastral (bipolar spindles with only one centrosome)	0.5	1.88	0	0
Anastral (bipolar spindles with no centrosomes)	0.5	4.89**	0.7**	0
Promiscuous MTs (MT spurs or interactions between spindles)	0	0.75	0.35	0.49
Multi MTOC (bipolar spindles with multiple MTOCs)	1.99	3.76	5.57	11.75**
Monopolar spindles	0	6.77***	0.35***	8.29***
Total	2.99	18.05	6.97	20.53
Cortical Divisions (Cycles 11–13)	n = 118	n = 104	n = 191	n = 175
Monopolar spindles	0.85	0	0.52	1.14
Promiscuous MTs (metaphase)	0	1.83	4.19	1.71
Promiscuous MTs (anaphase)	0	0	2.09	6.86*
Total	0.85	1.83	7.78	9.71
Total Defects	**3.84**	**19.88**	**14.75**	**30.24**

The following abbreviations are used: MT, microtubule; MTOC, microtubule-organizing center. n represents the number of spindles assayed from 30 (interior) or 3 (cortical) embryos. *p < 0.05; **p < 0.01; ***p < 0.001. (*klp61f* embryos are compared statistically to wild-type embryos; [WT] are compared statistically to *klp61f*; [3YF] are compared statistically to [WT].)

The incidence of promiscuous microtubules in [3YF];*klp61f*^{3} embryos, ~7%, was lower than the ~28% incidence in *dwee1* mutant embryos (Table 1) [11]. This could be because dWee1 acts through both KLP61F and nuclear/spindle positioning [11] to prevent collisions. Also, the presence of endogenous KLP61F might prevent a stronger effect by Myc-KLP61F^{3YF}. Because KLP61F forms tetramers [2, 4], Myc-KLP61F^{3YF} might complex with endogenous KLP61F. The resulting complex could be compromised in function and could also explain why Myc-KLP61F^{3YF} acts in a dominant-negative manner.

These results lead us to conclude that mutation of tyrosines in KLP61F, which are important for in vitro phosphorylation by dWee1, phenocopies spindle defects seen in *dwee1* mutant embryos in vivo. We suggest that in the absence of phosphorylation by dWee1, Myc-KLP61F^{3YF} is less functional. This would explain the failure of Myc-KLP61F^{3YF} to rescue monopolar spindles in *klp61f*^{3} embryos or to rescue polyploidy in *klp61f*^{3} mutant larvae. Reduced ability of KLP61F to crosslink and translocate along microtubules would result in loss of spindle bipolarity and chromosome segregation failure. In addition, a decreased ability of Myc-KLP61F^{3YF} to crosslink microtubules might lead to reduced organization within the spindle. Resulting microtubule spurs could then interact with neighboring spindles in a syncytium, especially during anaphase when spindles are no longer protected by actin furrows.

Multiple Roles for dWee1

Based on these and previous data, dWee1 appears to regulate mitosis in three ways during syncytial divisions: (1) it negatively regulates Cdk1 to time the entry into mitosis [10]; (2) it regulates γ-TuRC, perhaps indirectly, to position the mitotic spindle directly beneath the cortex [11]; and (3), it positively regulates KLP61F to preserve spindle integrity and prevent microtubule interaction between neighbors (Figure 4I). Regulation of KLP61F by dWee1, we propose, is important for bundling parallel and/or antiparallel microtubules and, consequently, generating a robust bipolar spindle. Without dWee1 regulation, KLP61F activity would be reduced, leading to

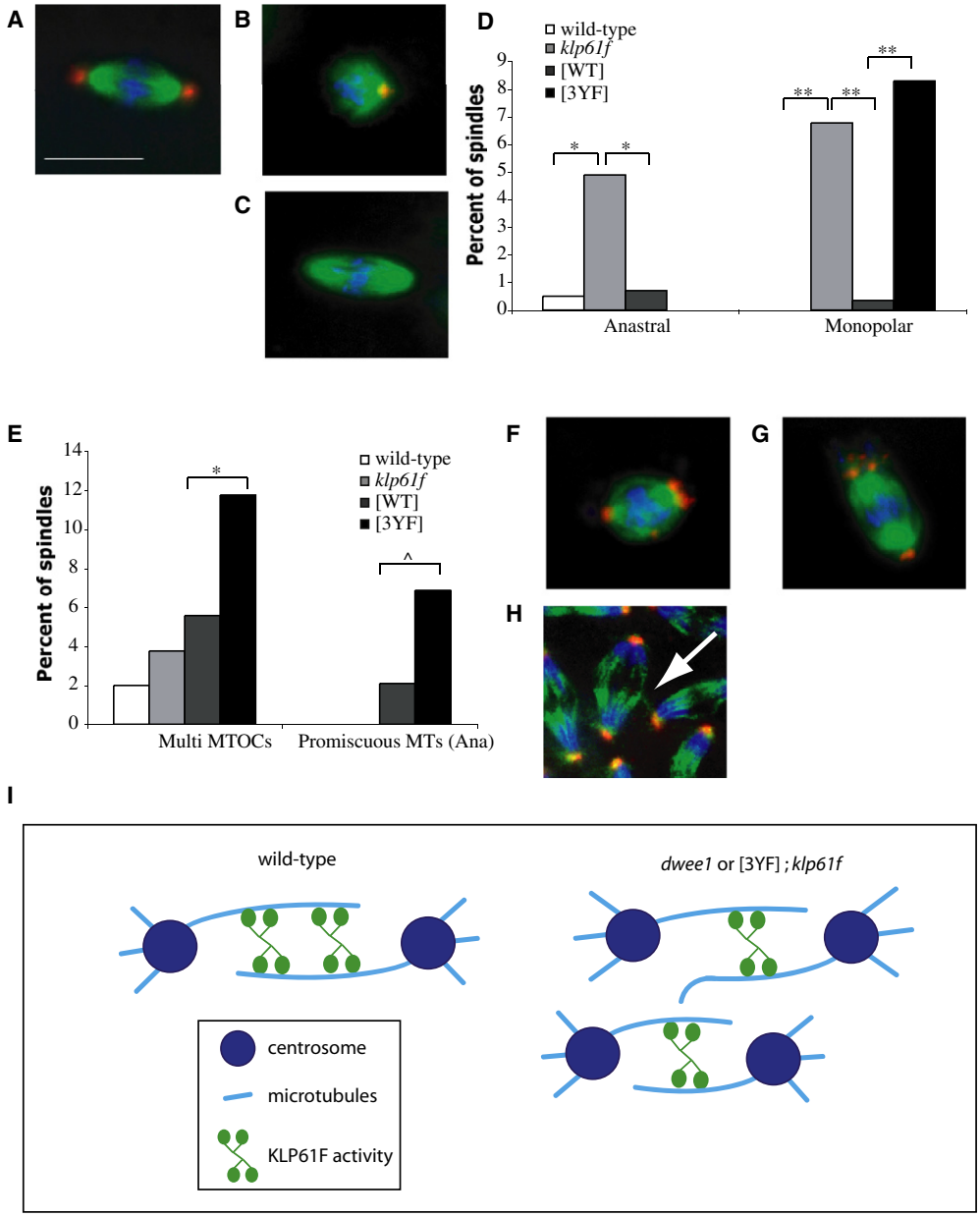

Figure 4. Mitotic Spindle Defects in Embryos Containing KLP61F^{WT} or KLP61F^{3YF}

(A–H) Embryos from mothers that were wild-type, *klp61f*^{3} heterozygous (*klp61f*), *klp61f*^{3} heterozygous with Myc-KLP61F^{WT} ([WT]), or *klp61f*^{3} heterozygous with Myc-KLP61F^{3YF} ([3YF]) were fixed and stained for DNA (blue), α-tubulin (green), and centrosomin (red). Representative images show a normal spindle (A) and defects significantly different among the genotypes: a monopolar spindle (B), an anastral spindle (C), spindles with multiple microtubule-organizing centers (F and G) from interior divisions, and a promiscuous microtubule interaction during anaphase in cortical divisions (H). Scale bar represents 5 μm. Defects in (A)–(C) and (F)–(H) are quantified in (D) and (E), respectively. See Table 1 for numeric quantification. ^p < 0.05; *p < 0.01; **p < 0.001.

(I) A model for regulation of KLP61F to maintain mitotic spindle integrity and prevent promiscuous microtubule interactions. dWee1 might regulate KLP61F activity to bundle parallel (not shown) and/or antiparallel microtubules to create a more robust spindle (left). Without dWee1 regulation, KLP61F activity is reduced on the spindle, leading to an unstable spindle with microtubule spurs. These microtubule spurs can then interact with neighboring spindles in a syncytium (spur-spindle interaction is not depicted). Reduced KLP61F activity on the spindle is depicted as reduced protein levels on the spindle, but it remains possible that similar levels of protein associate with the spindle but display reduced activity.

unstable spindles, microtubule spurs, and interaction between neighboring spindles in a syncytium.

Outside the syncytium, mitotic regulation by dWee1 also appears to be important, particularly during somatic cell cycles when the phosphoacceptor tyrosines of KLP61F are required for larval viability and chromosome segregation in neuroblasts. Consistent with this idea, dWee1 is also necessary to prevent mitotic defects in neuroblasts [10]. *dwee1*, however, appears dispensable for larval growth because homozygous or hemizygous *dwee1* mutants survive to adulthood, whereas Myc-KLP61F^{3YF} cannot support larval growth. We speculate that another kinase might partially substitute for dWee1 in the larva. Precedence for overlapping function between Wee1 and another kinase (Myt1) is seen in Cdk1 regulation [24].

Implications of Phosphorylation within the KLP61F Head Domain

Our studies show that the head domain of KLP61F is phosphorylated by dWee1. Regulation of a specific kinesin via phosphorylation in this domain might appear unlikely, considering that it is highly conserved among all kinesins. However, we found that modification by dWee1 might be specific to the KLP61F head domain because the KHC head domain was a poor substrate. Previous studies have shown that domains outside of the kinesin-5 head domain are also phosphorylated [25–28]. Cdk1 phosphorylates kinesin-5 on a threonine in the BimC box and is important for spindle localization of the motor [3, 26, 29]. Nek6, a NIMA (never in mitosis gene A)-related kinase, also phosphorylates kinesin-5 in the tail domain, possibly to regulate its function at the spindle poles [28]. The coiled-coil domain of *Xenopus* kinesin-5 is also phosphorylated on a nonconserved serine by Aurora kinase but does not appear to be important for spindle assembly [27, 29]. Whether and how these phosphorylation events affect activity of the head domain remains unknown. Direct regulation via phosphorylation within catalytic regions is not unheard of. Indeed, Wee1 kinases inhibit Cdk1 activity by phosphorylation in the ATP-binding domain [30]. The structure of KLP61F remains to be determined, but in the structure for human kinesin-5, the amino acid that correspond to Y207 of KLP61F is located near regions involved in nucleotide sensing [31] (Figure S4). The amino acid that corresponds to another potential phosphoacceptor, Y152, is near regions of KLP61F that are important for microtubule interaction [32] (Figure S4). While this work was under review, Menella et al. reported that *Drosophila* kinesin-13 is phosphorylated in the head domain and that this modification alters the protein's ability to depolymerize microtubules [33]. Exactly how phosphorylation in the head domain by dWee1 alters KLP61F activity remains to be investigated, but it is intriguing that two of the potential phosphoacceptor tyrosines may reside near regions important for kinesin activity.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01545-0.

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