# Cell cycle regulation of Greatwall kinase nuclear localization facilitates mitotic progression

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Cell division requires the coordination of critical protein kinases and phosphatases. Greatwall (Gwl) kinase activity inactivates PP2A-B55 at mitotic entry to promote the phosphorylation of cyclin B–Cdk1 substrates, but how Gwl is regulated is poorly understood. We found that the subcellular localization of Gwl changed dramatically during the cell cycle in *Drosophila*. Gwl translocated from the nucleus to the cytoplasm in prophase. We identified two critical nuclear localization signals in the central, poorly characterized region of Gwl, which are required for its function. The Polo kinase associated with and phosphorylated Gwl in this region, promoting its binding to 14-3-3ε and its localization to the cytoplasm in prophase. Our results suggest that cyclin B–Cdk1 phosphorylation of Gwl is also required for its nuclear exclusion by a distinct mechanism. We show that the nucleo-cytoplasmic regulation of Gwl is essential for its functions in vivo and propose that the spatial regulation of Gwl at mitotic entry contributes to the mitotic switch.

## Introduction

The molecular events driving the cell cycle are regulated by a complex network of kinases and phosphatases with cyclically ordered and specific activities. The cyclin-dependent kinase 1 (Cdk1) in complex with cyclin B triggers mitosis by promoting nuclear envelope breakdown (NEBD), chromosome condensation, and spindle assembly (Morgan, 2007). Many effector and regulatory proteins of mitosis are targets of cyclin B–Cdk1, and their phosphorylation must be reversed to allow mitotic exit. This dephosphorylation is thought to be largely achieved by protein phosphatase 2A (PP2A) in complex with its B-type regulatory subunits known as B55 in vertebrates and Twins (Tws) in *Drosophila* (Mayer-Jaekel et al., 1994; Mochida et al., 2009; Schmitz et al., 2010). In addition to Cdk1, several other kinases are required to coordinate the events of mitosis and cytokinesis. These include members of the Polo-like kinase and Aurora kinase families (Archambault and Glover, 2009; Carmena et al., 2009).

Greatwall (Gwl; MASTL in humans) was first discovered in *Drosophila* as an essential mitotic kinase (Yu et al., 2004; Archambault et al., 2007; Glover, 2012). *gwl* mutants show delays in chromosome condensation and chromosome segregation defects in larval neuroblasts, and similar phenotypes were observed in Gwl-depleted S2 cells (Bettencourt-Dias et al., 2004; Yu et al., 2004; Archambault et al., 2007). Gwl was then shown to be essential for mitosis by its participation in the positive feedback loop leading to full cyclin B–Cdk1 activation in *Xenopus* extracts (Yu et al., 2006). Strong evidence now indicates that Gwl antagonizes PP2A-B55 in its ability to dephosphorylate Cdk1 substrates in frogs, flies, and humans (Castilho et al., 2009; Vigneron et al., 2009; Burgess et al., 2010; Rangone et al., 2011; Wang et al., 2011). This function of Gwl was shown to be mediated by the endosulfine and Arpp19 homologous proteins in vertebrates, and by their sole orthologue, Endos, in *Drosophila*. In *Xenopus* extract, these proteins are phosphorylated by Gwl at mitotic entry to become inhibitors of PP2A-B55δ, thereby promoting the phosphorylated state of Cdk1 substrates (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). In *Drosophila*, mutations in *endos* rescue maternal-effect embryonic defects induced by a gain of Gwl function, and Gwl regulates Endos at a site conserved with *Xenopus* endosulfine and Arpp19 (Rangone et al., 2011). Therefore, the Gwl–Endos–PP2A–B55/Tws pathway appears strongly conserved (Glover, 2012; Lorca and Castro, 2013).

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Abbreviations used in this paper: Gwl, Greatwall; NEBD, nuclear envelope breakdown; NLS, nuclear localization signal; PP2A, protein phosphatase 2A; TWS, Twins; wt, wild type.



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The current model predicts that in order to mediate the regulation in PP2A-B55/Tws activity through M-phase, Gwl and/or Endos must be active at mitotic entry and inactive at mitotic exit. The molecular mechanisms of this regulation are unclear. Gwl has been shown to become activated and hyperphosphorylated at mitotic entry in *Xenopus* extracts (Yu et al., 2006). Recently, the kinase activity of Gwl has been proposed to be regulated by a noncanonical mechanism for the AGC family of kinases to which it belongs. This mechanism is thought to require the phosphorylation of Gwl in its C-terminal tail/linker site and binding of another kinase to a hydrophobic motif in the N-terminal lobe of Gwl (Vigneron et al., 2011). Another study in *Xenopus* identified three phosphorylation sites in Gwl that can increase its activity (Blake-Hodek et al., 2012). The identity of the kinases activating Gwl in vivo is uncertain, but strong evidence implicates cyclin B–Cdk1 and Gwl itself in this process (Yu et al., 2006; Vigneron et al., 2011; Blake-Hodek et al., 2012). Plx1 (*Xenopus* Polo) has been shown to phosphorylate Gwl (Yu et al., 2006; Peng et al., 2011; Vigneron et al., 2011) in *Xenopus* extracts, and this has been proposed to help Gwl drive reentry into mitosis in recovery from DNA damage (Peng et al., 2011). However, only very modest activation of Gwl was detected upon its phosphorylation by Polo, and a recent study failed to detect any effect of Polo phosphorylation on Gwl activity in vitro (Blake-Hodek et al., 2012). To what extent specific phosphorylation events contribute to regulate Gwl activity in vivo and whether other mechanisms come into play to regulate Gwl function is unknown. In this regard, Gwl possesses an intriguing, uniquely long protein segment in lieu of a T-loop within the kinase domain (Yu et al., 2004). Any segment of this region can be deleted with little effect on kinase activity in vitro (Blake-Hodek et al., 2012). The role of Gwl's central region remains completely unknown.

Here, we have explored how Gwl is regulated at the level of its subcellular localization in *Drosophila*. We show that Gwl is strongly nuclear in interphase, but becomes cytoplasmic and excluded from the nucleus just before mitosis. We found that Gwl's central region contains two nuclear localization signals (NLSs) required for the nuclear targeting of Gwl. Moreover, Polo associates with and phosphorylates Gwl in the same region. Our results suggest that Polo phosphorylation of Gwl allows its binding to 14-3-3$\varepsilon$, which promotes the relocalization of Gwl to the cytoplasm, where PP2A-Tws is largely localized. Cdk1 sites in the central region of Gwl are also required for its efficient nuclear exclusion in prophase. Importantly, we show that this spatial regulation of Gwl is essential in vivo and is required for normal mitotic progression.

## Results

### The localization of Gwl is cell cycle regulated

At the time of its identification as a mitotic kinase, Gwl was found to be a nuclear protein in interphase (Yu et al., 2004). However, how Gwl changes localization through the cell cycle was not investigated. We examined the localization of Gwl in *Drosophila* early embryos, where nuclei divide rapidly in a syncytium. By immunofluorescence, we found that Gwl is nuclear during interphase, but becomes mostly cytoplasmic in prophase, appearing excluded from nuclei before nuclear envelope fenestration, which becomes apparent later when microtubules cross the nuclear envelope (Fig. 1 A). Gwl gradually becomes more diffuse through the embryo during mitosis, but appears excluded again from daughter nuclei in telophase. We confirmed this dynamic localization pattern by time-lapse imaging of embryos coexpressing Gwl-GFP and H2Av-RFP (Fig. S1 A).

To examine if this localization pattern of Gwl is generally conserved in other cell types, we generated a D-Mel cell line stably expressing GFP-tagged Gwl. As seen in embryos, GFP-Gwl was nuclear in interphase, and became cytoplasmic and excluded from the nucleus in prophase, for ∼5 min just before NEBD (Fig. 1 B and Video 1). In addition, GFP-Gwl was enriched at the nuclear envelope in early prometaphase (Fig. 1 B and Video 1). Similar results were obtained with C-terminally tagged Gwl-GFP. Although immunofluorescence results have suggested the presence of MASTL at the centrosomes and the cytokinetic midbody in human cells (Burgess et al., 2010; Voets and Wolthuis, 2010), we did not observe these localizations for Gwl in *Drosophila*.

### Gwl contains two functional NLS motifs in its central region

We explored the mechanisms responsible for this dynamic localization of Gwl through the cell cycle. Gwl shows a strongly nuclear localization in interphase. To identify the region of Gwl responsible for its nuclear localization, we expressed Myc-tagged truncated forms of Gwl in D-Mel cells in culture and examined their localization. The region containing amino acid residues 400–675 was necessary and sufficient for nuclear localization (Fig. 2 A). We searched for potential nuclear localization signals (NLSs) in this region of the Gwl protein sequence. NLS motifs are often characterized by the presence of at least three consecutive positively charged residues (Lange et al., 2007). We found two potential NLS motifs (NLS1 and NLS2) conserved between several related species (Fig. 2 A and Fig. S2). Whereas mutation of each NLS individually affected Gwl's localization only partially (not depicted), mutation of both of these motifs was sufficient to disrupt the nuclear localization of Gwl in cells (Fig. 2 B) and in embryos (Fig. 2 C). Thus, the nuclear localization of Gwl depends on two NLSs in its central region. Importantly, nuclear localization is the first function assigned to the long central region of Gwl that interrupts its kinase domain.

### Nuclear localization of Greatwall is required for its function

We next tested the importance of the nuclear localization of Gwl for its biological function. To examine the effect of a mislocalization of Gwl on mitosis, we used cells in culture in which we expressed Gwl-GFP (wild type [wt] or NLS mutant) under the control of a copper-inducible promoter while depleting endogenous Gwl. RNAi against the coding sequence of Gwl led to its efficient silencing, but targeting untranslated regions of endogenous Gwl transcripts did not (unpublished data), precluding this potential strategy for the specific silencing of endogenous Gwl.

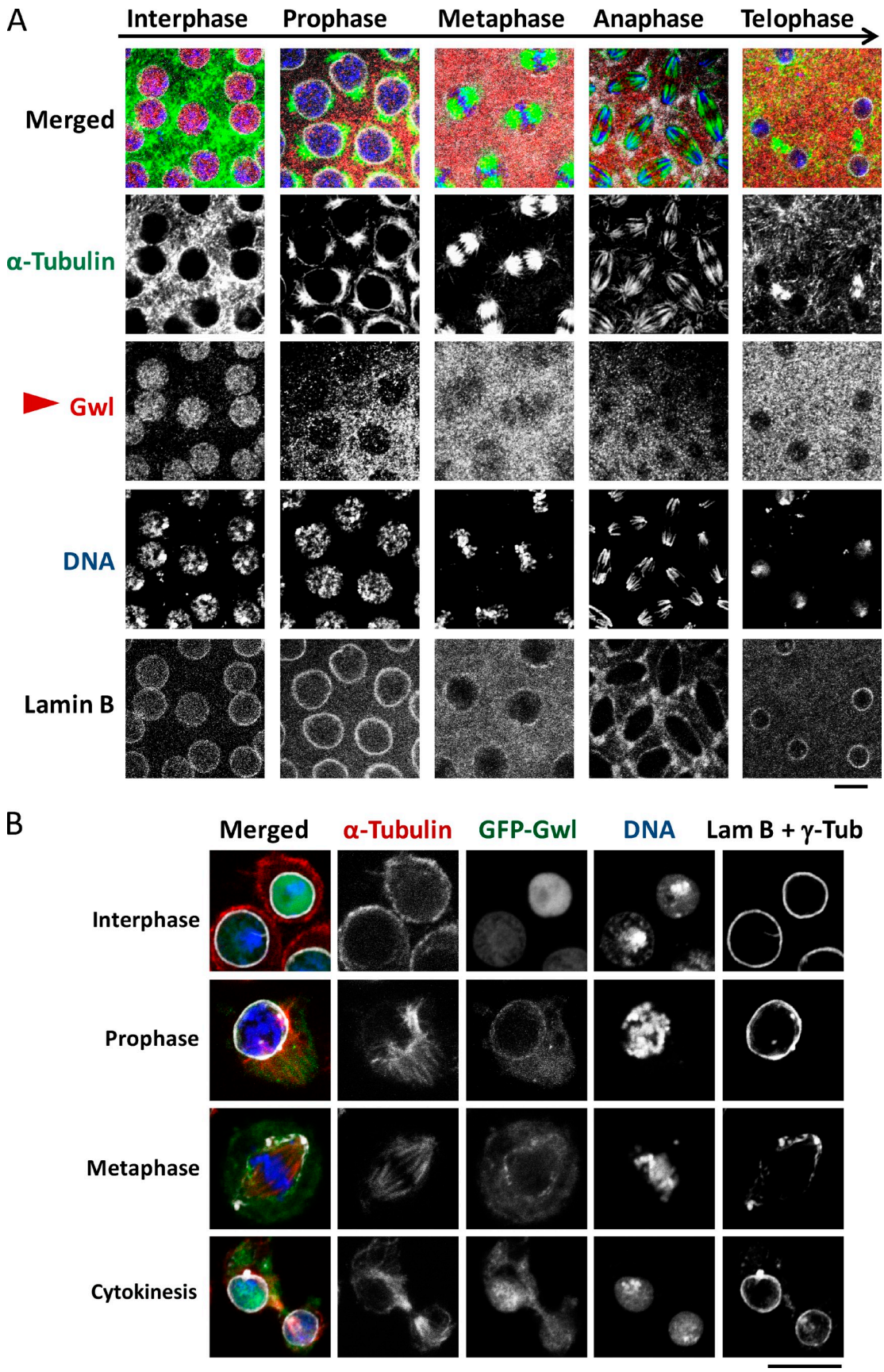


Figure 1. **The localization of Gwl is cell cycle regulated.** Immunofluorescence in fixed syncytial embryos (A) and D-Mel cells expressing GFP-Gwl (B) at different stages of the cell cycle. Note that in both panels, Gwl is nuclear in interphase and cytoplasmic in prophase, appearing excluded from nuclei. Bars, 10 μm.

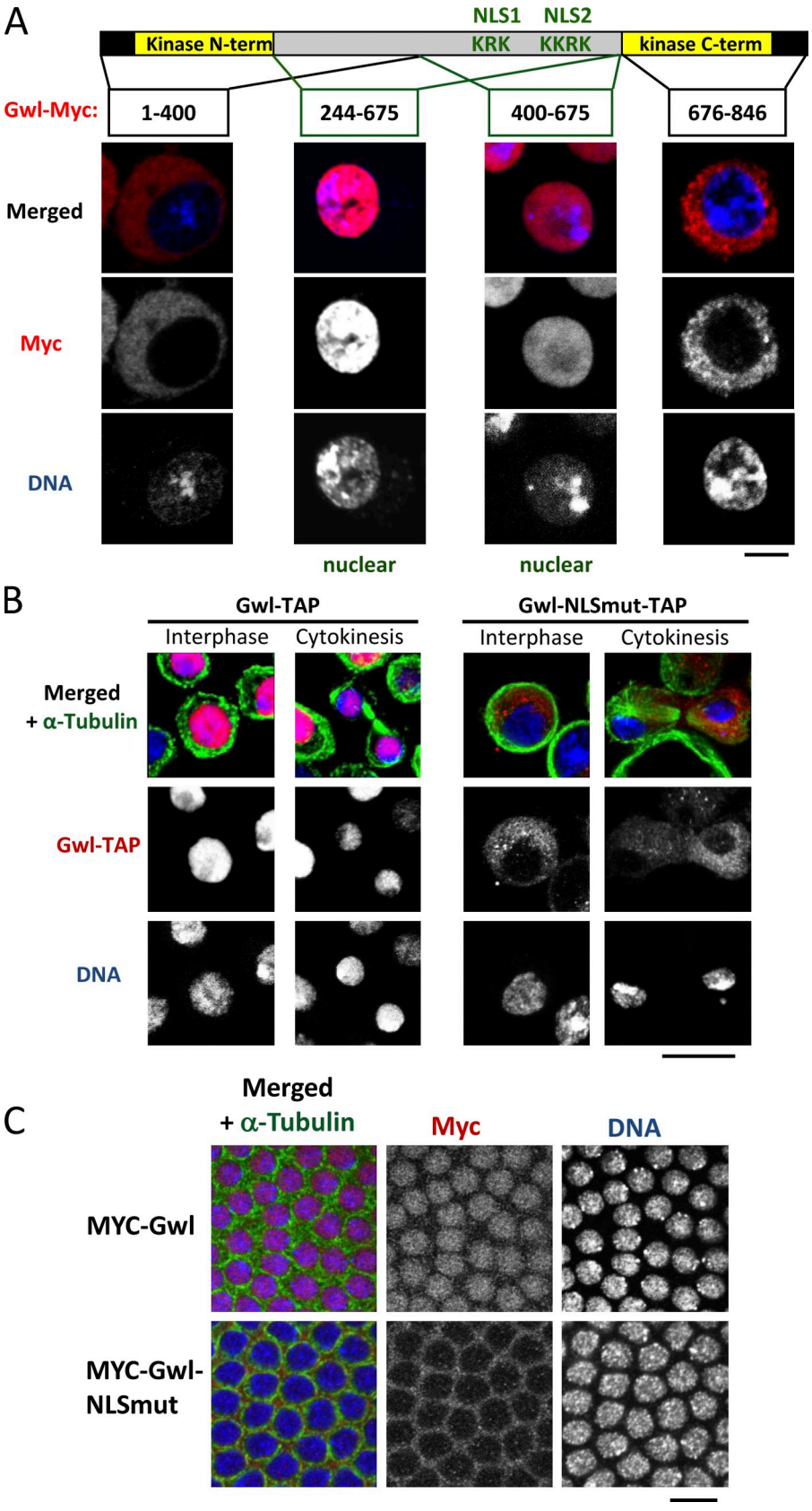


Figure 2. **Gwl contains two essential NLS motifs in its central region.** (A) The central region of Gwl is necessary and sufficient for nuclear localization. D-Mel cells expressing the indicated truncations of Gwl tagged with Myc were examined by immunofluorescence on a confocal microscope. Identified NLS motifs are shown. Bar, 5 µm. (B and C) Mutation of both NLSs (NLSmut = K518M, K520M [in NLS1] + K564M, R566L [in NLS2; see Fig. S2]) prevents the nuclear localization of Gwl. Immunofluorescence in D-Mel cells (B) and in syncytial embryos (C) expressing the indicated proteins. Bars, 10 µm.

Thus, we created Gwl cDNAs resistant to a dsRNA targeting the coding sequence by introducing conservative codon replacements. RNAi silencing of Gwl in D-Mel cells resulted in a marked increase in bipolar spindles with scattered chromosomes (Fig. 3, A–C), consistent with previous findings (Bettencourt-Dias et al., 2004; Archambault et al., 2007; Rangone et al., 2011).

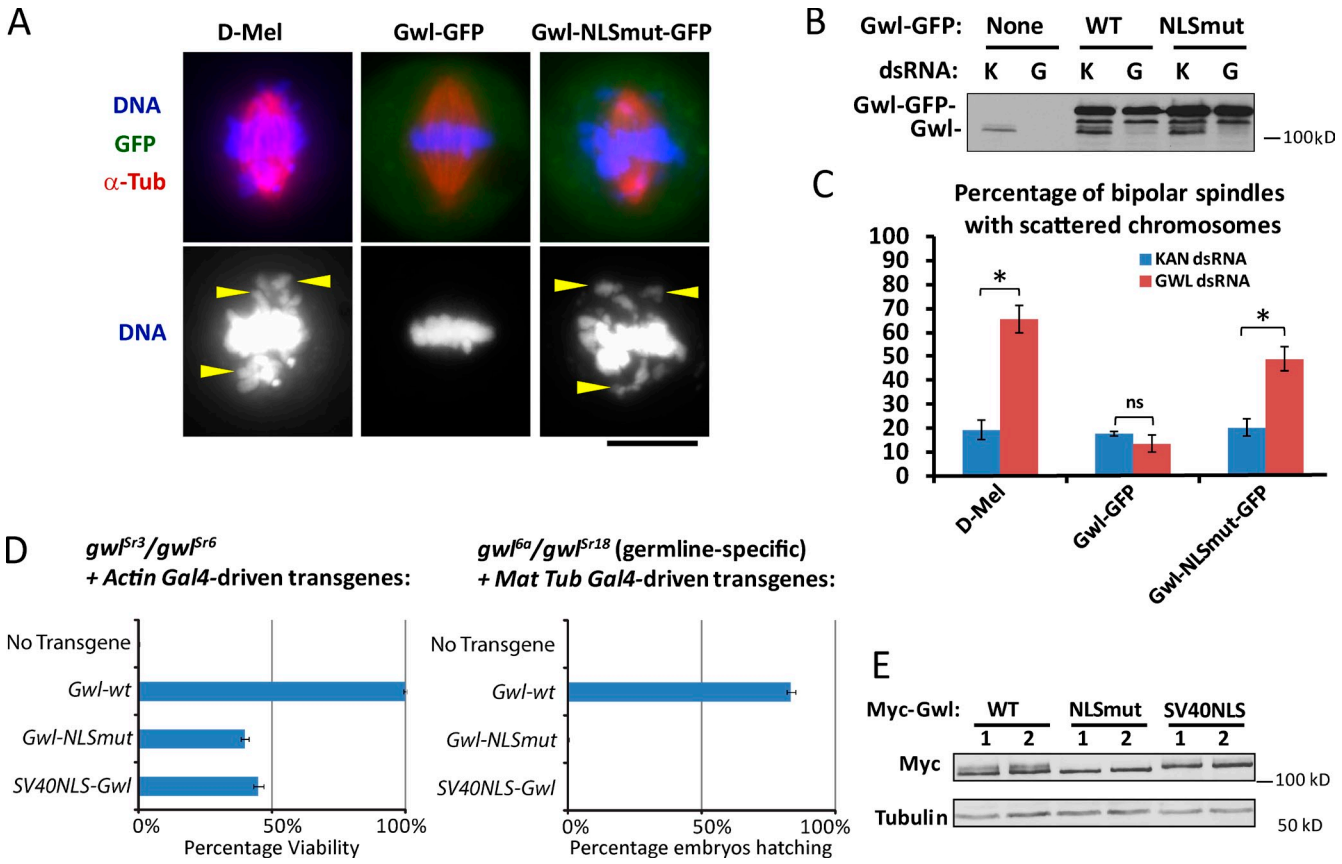


Figure 3. **Nuclear localization of Greatwall is required for its function.** (A–C) Nuclear localization of Gwl is required for its mitotic function in cells. Stable cell lines allowing the inducible expression of RNAi-resistant forms of Gwl-GFP (wt or NLSmut) were generated. Simultaneously with induction, D-Mel cells were transfected with dsRNA to deplete endogenous Gwl (G), or with a dsRNA against the bacterial *KAN* gene (K) as a control. After 4 d, cells were analyzed by immunofluorescence (A) and Western blotting for Gwl (B). Bar, 10 µm. (C) Quantification of cells with bipolar spindle that have scattered chromosomes (A, arrows). Results shown are averages of three independent experiments ± SEM. Between 30 and 50 cells were examined for each condition in each experiment. Asterisks: $P < 0.001$ after Student's *t* test. ns: nonsignificant. (D) The regulated localization of Gwl is essential for its functions in vivo. *UASp-Myc-Gwl* transgenes were expressed in *gwl* mutant flies as indicated and adult viability (left) and embryo hatch rates (right) were scored. Error bars: SEM For each construction, results from two independent transgenes were combined. (E) Western blot of ovaries expressing the indicated forms of *UASp-Myc-Gwl* driven by *Maternal α-Tubulin-Gal4-VP16* from flies used in D. Note that expression levels are similar for all transgenes and was approximately threefold higher than endogenous Gwl (Archambault et al., 2007).

Expression of Gwl-GFP rescued this phenotype completely (Fig. 3, A–C). However, rescue by expression of Gwl-NLSmut-GFP was only partial, despite a higher expression level than endogenous Gwl (Fig. 3, A–C). Therefore, the nuclear localization of Gwl in interphase is required for its full function in early mitosis.

We tested the importance of the nuclear localization of Gwl for its biological function in vivo. Gwl is an essential gene for *Drosophila* development. Null mutants die mostly as pharate adults (inside their pupal case), with very rare escapers that hatch with multiple morphological defects (Yu et al., 2004; Archambault et al., 2007). Expression of *UASp-Myc-Gwl* driven ubiquitously by *Actin-Gal4* completely rescued the viability of $gwl^{Sr3}/gwl^{Sr6}$ individuals (strongly hypomorphic mutants; Archambault et al., 2007). However, expression of *UASp-Myc-Gwl-NLSmut* rescued viability only incompletely in the same mutant background (Fig. 3 D, left). Moreover, flies rescued by Gwl-NLSmut were sterile and dissections revealed a complete lack of ovaries (not depicted). Therefore, the nuclear localization of Gwl is important for its functions during development.

Female germline function and early embryonic development require maternal supplies of Gwl (Archambault et al., 2007). To test if the nuclear localization of Gwl is also important in this context, we used a mutant allele of *gwl* that completely disrupts Gwl expression in the female germline only ($gwl^{Sr18}$), which leads to sterility due to defects in oogenesis and meiosis (Archambault et al., 2007). Expression of *UASp-Myc-Gwl* driven by the *Maternal α-Tubulin-Gal4-VP16* driver largely rescued female fertility assayed by the ability of their embryos to hatch, consistent with previous results (Archambault et al., 2007). In contrast, *UASp-Myc-Gwl-NLSmut* expression did not rescue fertility (Fig. 3, D [right] and E). Therefore, the nuclear localization of Gwl is important for its functions during oogenesis and early embryogenesis. Phenotypic analysis of eggs expressing Gwl-NLSmut was complicated by the fact that the very few eggs laid were abnormally fragile and broke during the fixation procedure.

Interestingly, fusing Gwl to a strong NLS from SV40 (Kalderon et al., 1984) reduced its ability to rescue the viability and fertility of *gwl* mutants (Fig. 3, D and E). This construction

weakened Gwl's ability to relocalize efficiently to the cytoplasm in prophase (Video 2). These results strongly suggest that Gwl needs to access both the nucleus and the cytoplasm in an orderly and timely manner before NEBD in order to fulfill its essential functions in vivo.

### Polo kinase interacts with and phosphorylates Gwl

To identify proteins that could contribute to regulate Gwl directly, we purified Gwl-PrA from transgenic early embryos and analyzed associated proteins by mass spectrometry. We detected peptides from the Polo kinase in the Gwl-PrA purification products, but not in purifications of other bait proteins under the same conditions (unpublished data). This result suggested that Polo interacts specifically with Gwl. This was confirmed by anti-Polo Western blot on Gwl-PrA purification products (Fig. 4 A). We also validated this result with the copurification of Gwl-PrA and Polo-Myc in embryos (Fig. 4 B). The interaction of Polo with several of its substrates has been shown to require specific residues in its noncatalytic Polo-box domain (PBD) that engage in contacts with a prephosphorylated motif in the target (Elia et al., 2003; Park et al., 2010). Mutation of these residues in Polo did not abolish its interaction with Gwl (Fig. 4 C), suggesting that prior phosphorylation of Gwl is not required for interaction with Polo. Other targets of Polo have been shown to engage in interactions that do not follow the phospho-priming model (Archambault et al., 2008; Park et al., 2010; Bonner et al., 2013).

To determine the region of Gwl that mediates its interaction with Polo, we expressed Myc-tagged truncations of Gwl in D-Mel cells, and tested which ones copurified with coexpressed Polo-PrA. We found that the central region of Gwl, containing the functional NLSs, is sufficient for the association with Polo (Fig. 4 D). Thus, Polo can associate with the central region of Gwl. In addition, we detected a weaker association between Polo and the N-terminal half of the kinase domain of Gwl (not depicted), consistent with a previous study in *Xenopus* (Peng et al., 2011). It is therefore possible that two regions of Gwl can interact with Polo.

We hypothesized that Polo could regulate Gwl. It has been reported that Plx1, the *Xenopus* orthologue of Polo, can phosphorylate *Xenopus* Gwl in vitro, but the physiological relevance of this observation was unclear because only a minor effect or no effect on Gwl kinase activity could be detected (Yu et al., 2006; Peng et al., 2011; Vigneron et al., 2011; Blake-Hodek et al., 2012). We found that *Drosophila* Polo can phosphorylate *Drosophila* Gwl in vitro (Fig. 4 E). Furthermore, overexpression of Polo in embryos induces an upshift in the electrophoretic mobility of Gwl (Fig. 4 F), suggesting that Polo regulates Gwl phosphorylation in vivo. However, the effect of Polo overexpression on Gwl could have been an indirect consequence of alterations in cell cycle dynamics. Thus, we conducted an experiment in noncycling embryo extracts. We noticed that Gwl gradually collapsed to faster migrating forms in these extracts when incubated on ice (Fig. 4 G). This electrophoretic change of Gwl was partially reversed when extracts were incubated in the presence of okadaic acid, a phosphatase inhibitor, likely reflecting hyperphosphorylation. Addition of the Polo inhibitor BI2536 to the extracts abrogated this upshift, further suggesting that Polo phosphorylates Gwl. The remaining mobility shift observed is likely to be attributable in part to the known phosphorylation of Gwl by cyclin B–Cdk1 (Yu et al., 2006).

We used a similar assay in cultured cells to map the region of Gwl that is targeted by Polo. The central region alone ($Gwl_{245–675}$) shifted upon okadaic acid treatment, while the N-terminal and C-terminal regions, encoding for the core kinase domain did not shift (Fig. 5 A). Moreover, the mobility shift was reduced when cells were treated with the Polo inhibitor BI2536. Altogether, our results strongly suggest that Polo phosphorylates Gwl in its central region in vivo. Mass spectrometry analysis of Gwl after phosphorylation by Polo in vitro identified 32 sites, 20 of which are inside the central region of Gwl (Fig. S3 A). Altogether, our results suggest that Polo interacts with Gwl in its central region to phosphorylate it at multiple sites (Fig. 4 H).

### Polo activity promotes the cytoplasmic localization of Gwl in prophase

As shown above, the nuclear localization of Gwl relies on two NLS motifs located in its central region, and this region associates with and is phosphorylated by Polo. We hypothesized that the phosphorylation of Gwl by Polo could regulate its subcellular localization. To test this possibility, we overexpressed Polo in the syncytial embryo and analyzed the localization of Gwl by immunofluorescence. We found that overexpression of Polo or constitutively active $Polo^{T182D}$ partially abrogated the nuclear enrichment of Gwl (Fig. 5 B). Overexpression of $Polo^{T182D}$ in cultured cells also led to an increase in cytoplasmic localization of Gwl (Fig. 5 C). Phosphatase inhibition by short treatments with okadaic acid increased the cytoplasmic localization of Gwl, further suggesting that Gwl phosphorylation promotes its cytoplasmic localization (Fig. S4). As expected, simultaneous inhibition of Polo with BI2536 partially restored the nuclear localization of Gwl. These results strongly suggest that Polo phosphorylation of Gwl promotes its cytoplasmic localization.

To test if Polo activity is required for the nuclear exclusion of Gwl in prophase, we imaged mitotic entry in GFP-Gwl cells after inhibition of Polo with BI2536. Although control cells always excluded Gwl from the nucleus in prophase (in 20/20 cells filmed), cells treated with BI2536 entered mitosis and never showed nuclear exclusion of GFP-Gwl in prophase (in 18/18 cells filmed; Fig. 5 D). Instead, GFP-Gwl gradually spread into the cytoplasm in mitotic cells, when the nuclear envelope breaks down. Although control cells completed cell division in less than 2 h, Polo-inhibited cells failed to divide even after several hours. Therefore, Polo activity is required for the nuclear exclusion of Gwl in prophase.

Our mapping of the Polo phosphorylation sites in Gwl in vitro resulted in a large number of sites detected. Interestingly, these sites tended to be clustered in the central region of Gwl, comprising its NLSs (Fig. S3). We tested the effect of mutating Polo consensus sites in Gwl's central region on the localization of Gwl. Alanine residues were introduced instead of serine or threonine residues at all sites corresponding to the minimal Polo phosphorylation motif (E/N/D)-X-(S/T) (Santamaria et al., 2010) in the central region of Gwl, around its NLSs. This Gwl-PoloA

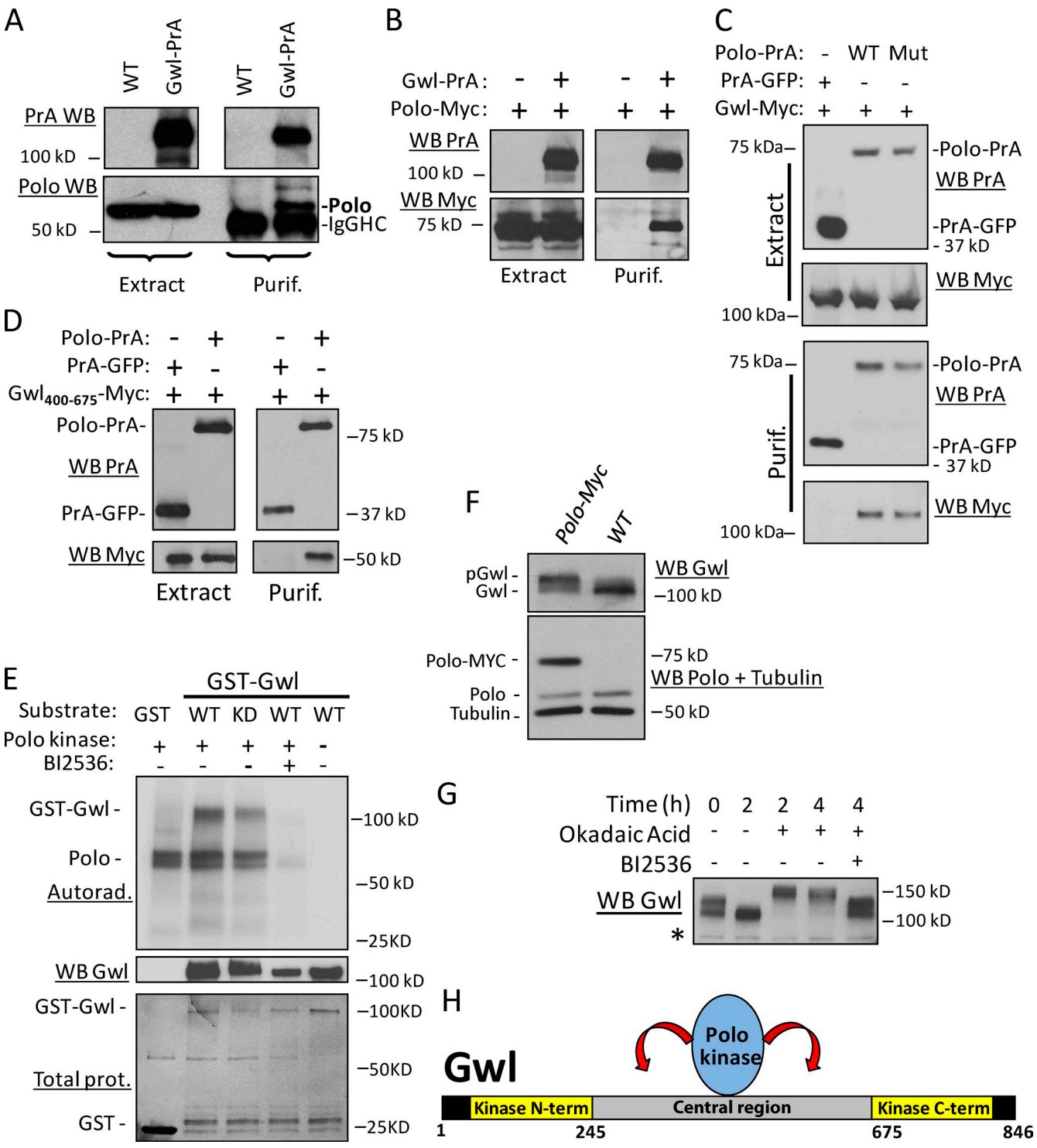


Figure 4. **Polo interacts with and phosphorylates Gwl.** (A) Polo is detected by Western blot in a Gwl-PrA purification product from embryos. (B) Polo-Myc is copurified with Gwl-PrA from transgenic embryos. (C) The association of Polo with Gwl does not depend on its canonical phospho-binding activity. D-Mel cells expressing the indicated proteins were used in PrA affinity purifications followed by Western blots. Mut = Polo W395F, H538A, K540A. (D) The central region of Gwl associates with Polo. Experiment done as in C. (E) Polo phosphorylates Gwl in vitro. KD: GST-Gwl-K87R, kinase-dead. BI2536 is a Polo inhibitor that was used as a control. (F) Moderate overexpression of Polo-Myc in embryos induces hyperphosphorylation of Gwl. (G) Gwl hyperphosphorylation depends on Polo in embryo extracts. After lysis, extracts were incubated as indicated. Okadaic acid induces an upshift in the mobility of Gwl that is abrogated by BI2536 (200 nM). (H) Model: Polo interacts with Gwl in its central region and phosphorylates multiple sites.

mutant was markedly less phosphorylated by Polo compared with Gwl-wt in vitro (Fig. S3 B). Remarkably, Gwl-PoloA-GFP failed to be excluded from the nucleus in prophase for 30/30 mitotic entry events examined (compare Fig. 6 A with 6 B, and Video 3 with Video 4), further supporting the idea that Polo phosphorylation of Gwl is required for its nuclear exclusion.

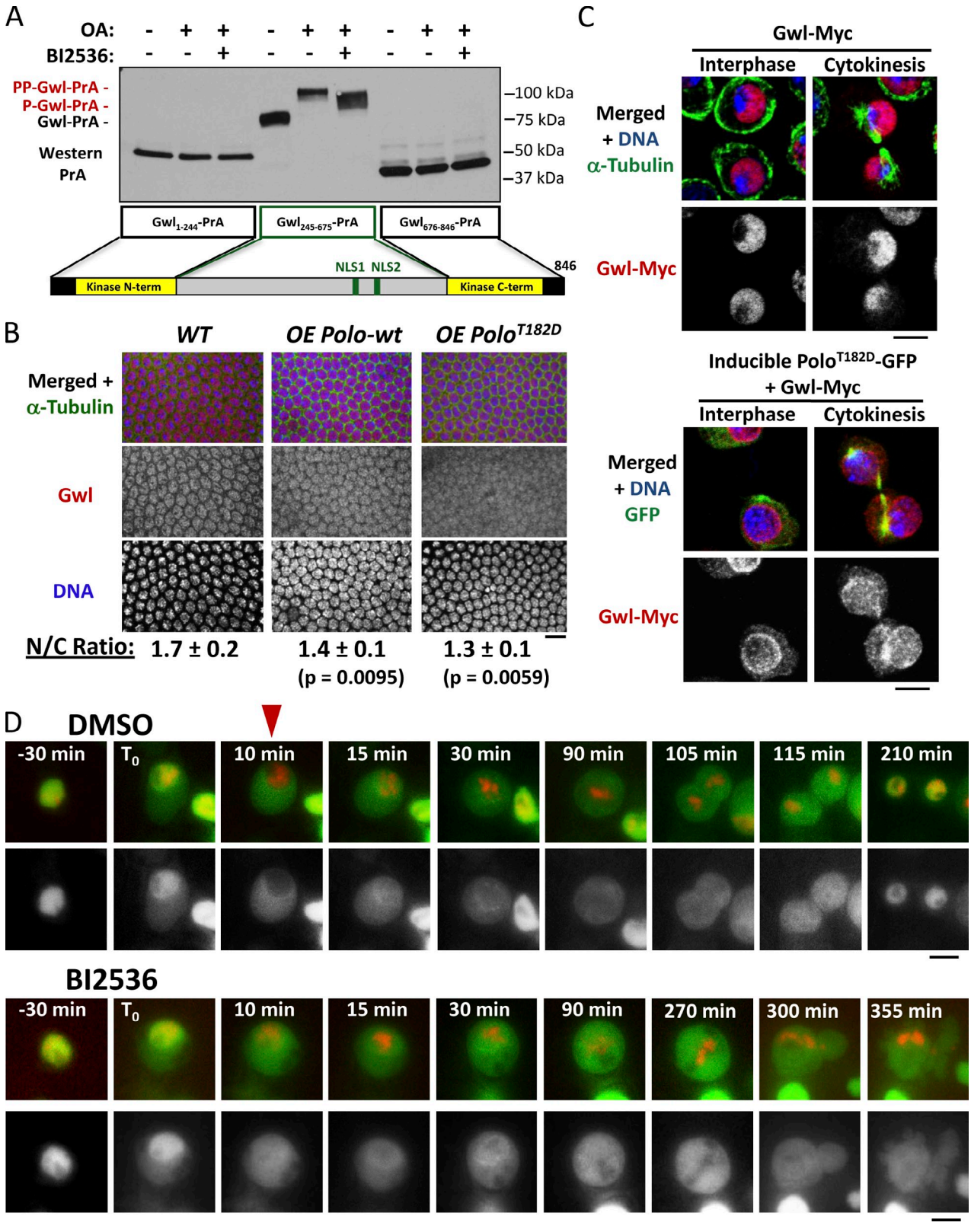


Figure 5. **Polo activity promotes the cytoplasmic localization of Gwl in prophase.** (A) Phosphorylation of Gwl in its central region depends on Polo. (B) Increasing Polo activity induces a more cytoplasmic localization of Gwl in syncytial embryos. P values are from a paired *t* test. (C) Increasing Polo activity induces a more cytoplasmic localization of Gwl in cultured cells. Bars, 5 µm. (D) Time-lapse microscopy of D-Mel cells expressing GFP-Gwl and H2Av-mRFP. In control, DMSO-treated cells, GFP-Gwl is excluded from the nucleus in prophase (red arrowhead). In cells treated with BI2536 (50 nM) to inhibit Polo, GFP-Gwl is never excluded from the nucleus and becomes diffuse throughout the cell in mitosis. $T_0$ was set as the time when cytoplasmic GFP-Gwl first begins to appear. Bars, 5 µm.

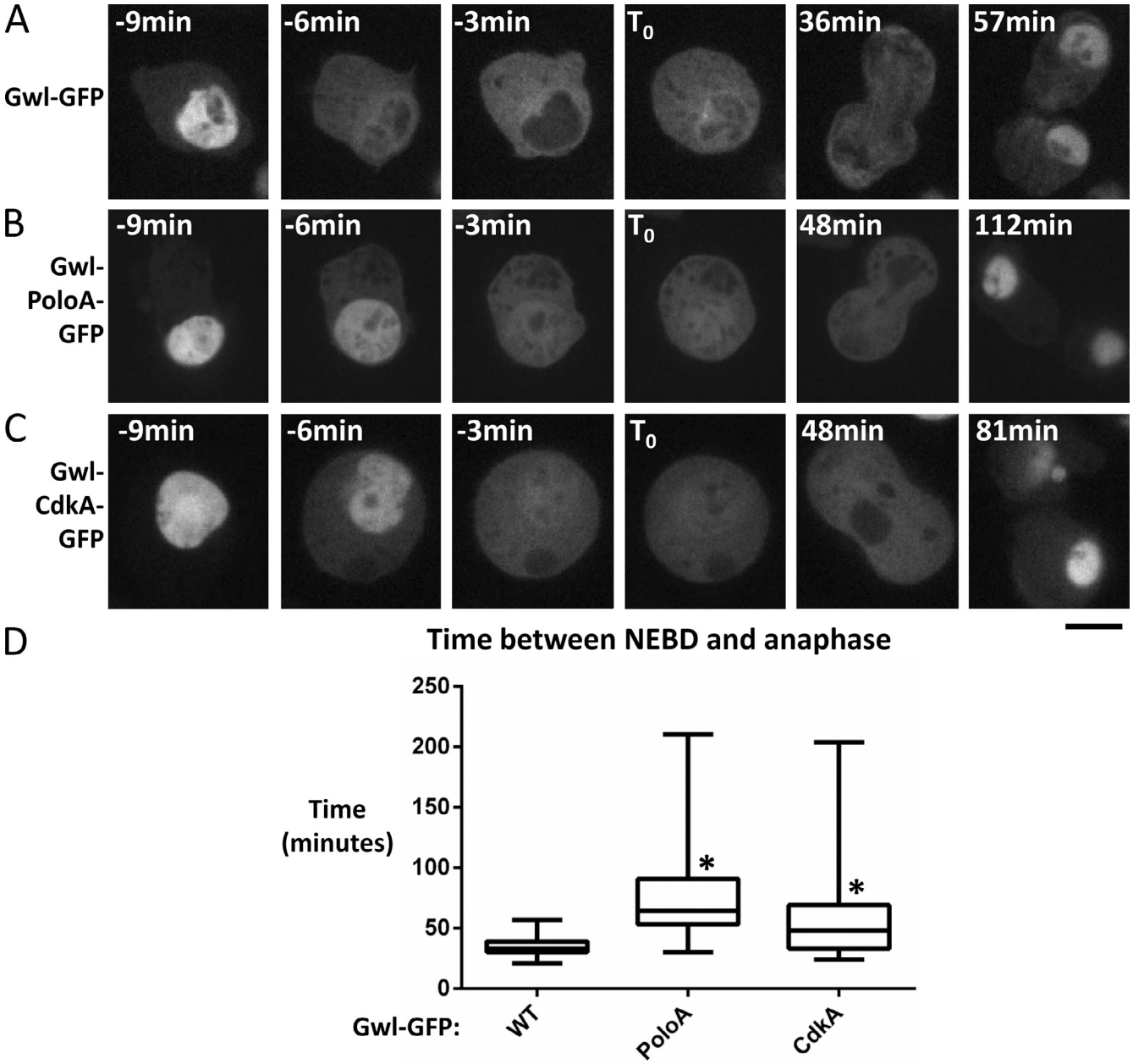


Figure 6. **Exclusion of Gwl from the nucleus in prophase requires Polo and Cdk consensus sites and is required for timely mitotic progression.** Expression of Gwl-GFP wt, PoloA, or CdkA was induced and cells were filmed one day later. (A) Gwl-GFP is nuclear in interphase and becomes cytoplasmic and excluded from the nucleus in prophase. After NEBD ($T_0$), Gwl-GFP becomes diffuse throughout the cell and returns to nuclei during cytokinesis. Bar, 5 µm. (B and C) Mutation of Polo or Cdk consensus sites in the central region of Gwl-GFP prevents its nuclear exclusion in prophase. (D) Expression of Gwl-PoloA-GFP or Gwl-CdkA-GFP extends early mitosis. The time between estimated NEBD (loss of round outline of NE) and anaphase (cell elongation) was measured and shown in a box plot (wt: *n* = 37 cells; PoloA: *n* = 26 cells; CdkA: *n* = 67 cells). Asterisks: $P < 0.001$ after Student *t* test.

### 14-3-3ε collaborates with Polo to promote the cytoplasmic localization of Gwl

Because a gain of Polo function in interphase led only to a partial relocalization of Gwl to the cytoplasm (Fig. 5), we hypothesized that other factors were required for this process to occur efficiently in prophase. 14-3-3 proteins are conserved phosphoserine/phosphothreonine-binding proteins that have been implicated in the cytoplasmic retention of several factors including regulators of mitotic entry such as Cdc25C (Gardino and Yaffe, 2011). Two 14-3-3 family members exist in *Drosophila*: 14-3-3ε and 14-3-3ζ. We tested if Gwl could interact with 14-3-3 proteins, as potential regulators of Gwl localization. Recombinant GST-14-3-3ε or GST-14-3-3ζ could pull down Gwl-Myc from a cell extract (Fig. 7 A). In *Drosophila* cells in culture, Gwl-Myc could be copurified with both 14-3-3ε and 14-3-3ζ fused to protein A (Fig. 7 B). In both assays, K-to-E mutations that weaken the phospho-binding activities of 14-3-3 proteins decreased their association with Gwl (Zhang et al., 1997). These results suggest that 14-3-3 proteins interact with phosphorylated Gwl.

We tested if 14-3-3 proteins could collaborate with Polo to promote the cytoplasmic localization of Gwl. We found that coexpression of 14-3-3ε and Polo$^{T182D}$ led to a marked increase

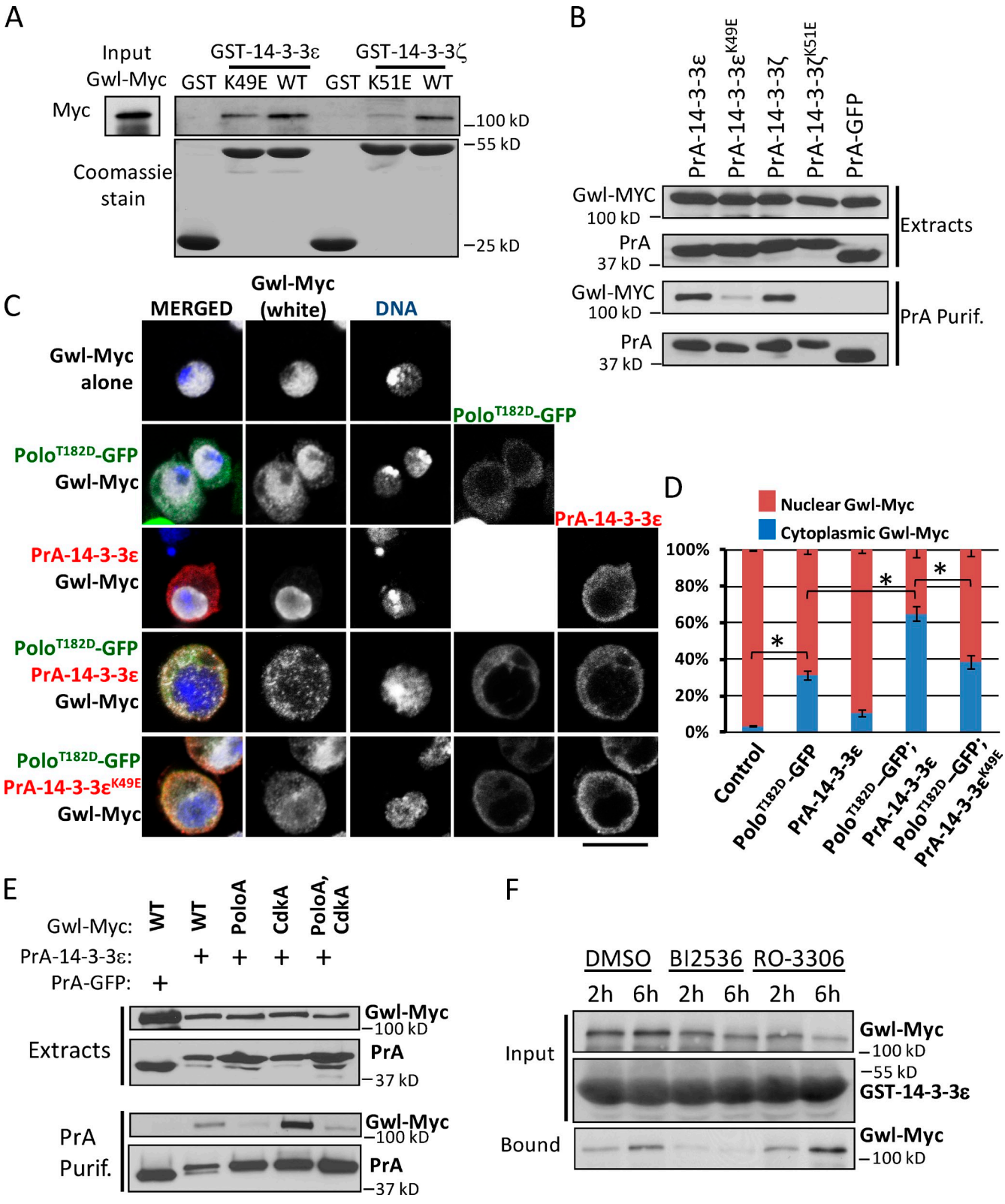


Figure 7. **14-3-3ε collaborates with Polo to promote the cytoplasmic localization of Gwl.** (A) Gwl is copurified with 14-3-3 proteins in a GST pulldown. An extract from D-Mel cells expressing Gwl-Myc was incubated with GST fusions proteins on Sepharose. (B) Gwl is copurified with 14-3-3 proteins from cells in culture. Cells coexpressing Gwl-Myc and Protein A fusion proteins as indicated were used in PrA affinity purifications. (C and D) Overexpression of 14-3-3ε enhances the cytoplasmic localization of Gwl induced by a gain of Polo function. (C) Examples of cells analyzed by immunofluorescence. Bar, 10 µm. (D) Quantification. For each condition, the cytoplasmic and nuclear fluorescence of Gwl-Myc was measured for multiple interphase cells taken randomly. Error bars: SEM. Asterisks: $P < 0.001$ after Student's *t* test. (E) Polo consensus sites but not Cdk consensus sites in the central region of Gwl are required for its interaction with 14-3-3ε. Cells expressing the indicated proteins were used in PrA affinity purifications followed by Western blots. (F) Treatment of cells with a Polo inhibitor (BI2536, 200 nM) but not a Cdk1 inhibitor (RO-3306, 10 µM) abrogated the interaction of Gwl-Myc with 14-3-3ε in a GST pulldown.

in cytoplasmic localization of Gwl-Myc in interphase cells, compared with the expression of Polo$^{T182D}$ or 14-3-3ε alone. This effect was abrogated by the K49E mutation in 14-3-3ε, suggesting that its phospho-binding activity is required for its effect on Gwl localization (Fig. 7, C and D). Moreover, the ability of Gwl to interact with 14-3-3ε depended on the presence of the Polo consensus sites in the central region of Gwl (Fig. 7 E). Moreover, cell treatment with the Polo inhibitor BI2536 weakened the interaction of Gwl with 14-3-3ε in a GST pulldown (Fig. 7 F). Together, these results strongly suggest that 14-3-3ε can bind Gwl after its phosphorylation by Polo, leading to the cytoplasmic retention of Gwl.

### Cdk1 may contribute to regulate Gwl localization

Like Polo, Cdk1 plays many important roles in mitosis by targeting several substrates. Moreover, Cdk1 has been shown to activate Gwl kinase activity (Yu et al., 2006; Vigneron et al., 2011; Blake-Hodek et al., 2012). To test if Cdk1 could regulate Gwl localization, we mutated into alanine residues six minimal CDK consensus sites ((S/T)-P) in the central region of Gwl. Gwl-CdkA was less efficiently phosphorylated by cyclin B–Cdk1 in vitro, compared with Gwl-wt (Fig. S3 C). Interestingly, time-lapse imaging revealed that Gwl-CdkA-GFP is not excluded from the nucleus in prophase (Fig. 6 C and Video 5, in 39/39 cells examined). These results suggest that phosphorylation of Gwl by cyclin B–Cdk1 is required for its nuclear exclusion in prophase. However, mutation of the Cdk sites in Gwl did not affect its ability to interact with 14-3-3ε (Fig. 7 E), and cell treatment with a Cdk1 inhibitor did not weaken Gwl's ability to interact with 14-3-3ε in the GST pulldown (Fig. 7 F). These results suggest that Cdk phosphorylation of Gwl affects its localization independently from the Polo/14-3-3 pathway.

### Failure to exclude Gwl from the nucleus leads to delays in mitosis

The failure in nuclear exclusion of Gwl observed for the Polo and CDK phosphorylation site mutants allowed us to ask if the nuclear exclusion of Gwl in prophase was required for normal mitosis. Strikingly, inducing the expression of Gwl-PoloA-GFP or Gwl-CdkA-GFP delayed the onset of anaphase relative to the time of NEBD (approximated by the loss of a visible round nuclear outline; Fig. 6 D). Although this time averaged 34 min in cells expressing Gwl-GFP, it extended to averages of 75 min in cells expressing Gwl-PoloA-GFP and 55 min in cells expressing Gwl-CdkA-GFP. In addition, this time was much more variable in cells expressing Gwl-PoloA-GFP and Gwl-CdkA-GFP, with some cells incurring delays of over 200 min (Fig. 6 D). Because endogenous Gwl was also expressed in those cells, these results strongly suggest a requirement for the nuclear exclusion of Gwl in prophase, rather than a simple requirement for the presence of Gwl in the cytoplasm.

### Excessive Polo activity in Gwl-compromised embryos leads to defective mitotic entry

We asked if Polo or cyclin B–Cdk1 could modify Gwl function in the cell cycle in syncytial embryos. Expression of only half the normal amount of Gwl in embryos laid by mothers heterozygous for a *gwl* null allele (*gwl*$^{6a}$) did not prevent embryos from hatching. However, overexpression of Polo in these *gwl*-compromised embryos markedly decreased their hatching rate, while moderate overexpression of Polo alone had only a moderate effect on embryo hatching (Fig. 8, A and B). Thus, Polo appears to modify Gwl activity in this genetic assay.

We examined the development defects in these embryos by immunofluorescence. In embryos expressing half the normal dose of Gwl and overexpressing Polo, we found that a significant fraction of nuclei failed to enter mitosis when the majority of nuclei were already in prometaphase or metaphase (Fig. 8 C). The defective nuclei were characterized by the absence of a mitotic spindle, a very strong lamin staining, and undercondensed chromosomes (Fig. 8 C). Such nuclei were also observed, albeit at a lower frequency, in embryos from *gwl*-heterozygous mothers. Nuclei either did or did not enter mitosis, and intermediate phenotypes were seldom seen, consistent with a stochastic failure to trigger the bi-stable mitotic switch in which Gwl is implicated (Domingo-Sananes et al., 2011). Similar observations were reported after injection of cyclin B dsRNA in syncytial embryos (McCleland et al., 2009). Expression of constitutively active Polo$^{T182D}$ was already toxic by itself, and was even more harmful to embryos from *gwl*-heterozygous mothers than Polo$^{wt}$, whereas Polo$^{T182A}$ had no effect on embryonic viability (unpublished data). We conclude that Polo kinase activity can regulate the essential function of Gwl in triggering mitotic entry. Overexpression of cyclin B in embryos from *gwl*$^{6a}$/+ mothers did not decrease their hatching rate (unpublished data), suggesting that Polo levels are more critical than cyclin B levels to regulate Gwl activity during embryonic cell cycles.

## Discussion

### A new level of regulation of Gwl in the cell cycle

We have shown that Gwl is regulated in the cell cycle at the level of its subcellular localization. From a strongly nuclear localization in interphase, Gwl becomes largely cytoplasmic and excluded from the nucleus in prophase, before NEBD. Our results indicate that Gwl needs to access the nucleus in interphase and translocate to the cytoplasm starting in prophase for proper cell cycle progression.

Based on our findings and existing knowledge on the factors involved, we can propose a mechanistic model for the role of Gwl's spatial regulation (Fig. 9). Nuclear sequestration of Gwl could facilitate its activation by cyclin B–Cdk1 in the nucleus at mitotic entry. Phosphorylation of cyclin B by cyclin B–Cdk1 has been shown recently to promote its nuclear import in prophase (Santos et al., 2012), and increased concentration of cyclin B in the nucleus further stimulates positive feedback loops leading to full cyclin B–Cdk1 activation (Lindqvist et al., 2009; Gavet and Pines, 2010a,b; Santos et al., 2012). In *Xenopus*, cyclin B–Cdk1 has been proposed to be at least partly responsible for Gwl activation (Yu et al., 2006; Vigneron et al., 2011; Blake-Hodek et al., 2012), but a delay has been observed between Cdk1 activation and Gwl activation in starfish oocytes

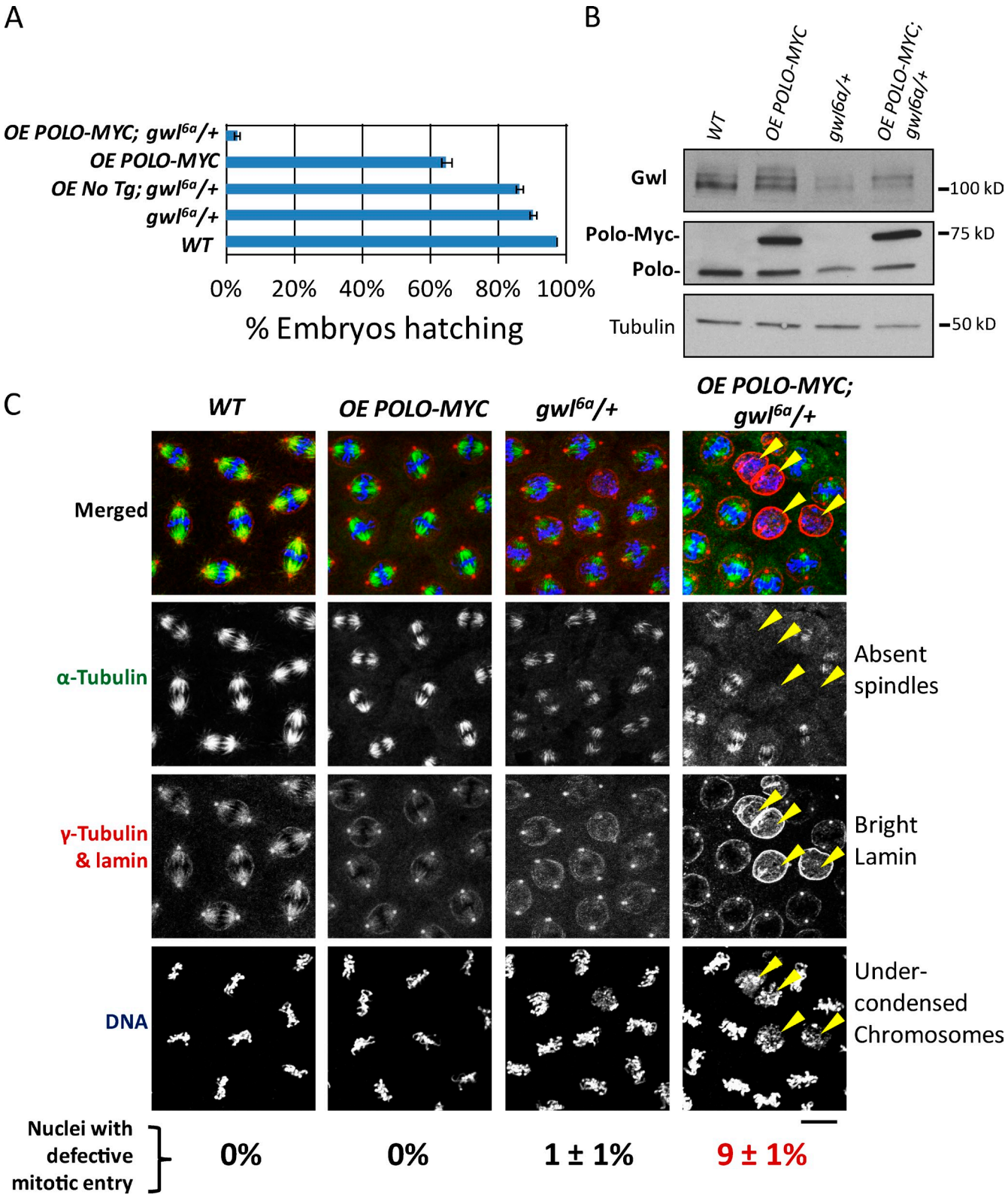


Figure 8. **Misregulation of Gwl by Polo interferes with cell cycle progression.** (A) Overexpression of Polo in syncytial embryos compromised for *gwl* is lethal. Expression of *UASp-Polo-Myc* was driven by *Maternal α-Tub Gal4-VP16* (OE: overexpression). No Tg: no Polo transgene. Error bars: SEM. (B) Western blot analysis of embryos analyzed in A. (C) Phenotypes of embryos where a majority of nuclei are in metaphase. Defects in mitotic entry are observed in embryos from mothers heterozygous for *gwl*$^{6a}$ where Polo is overexpressed. Defective nuclei (yellow arrowheads) are characterized by the absence of a spindle, bright lamin staining, and undercondensed chromosomes (quantification is at the bottom).

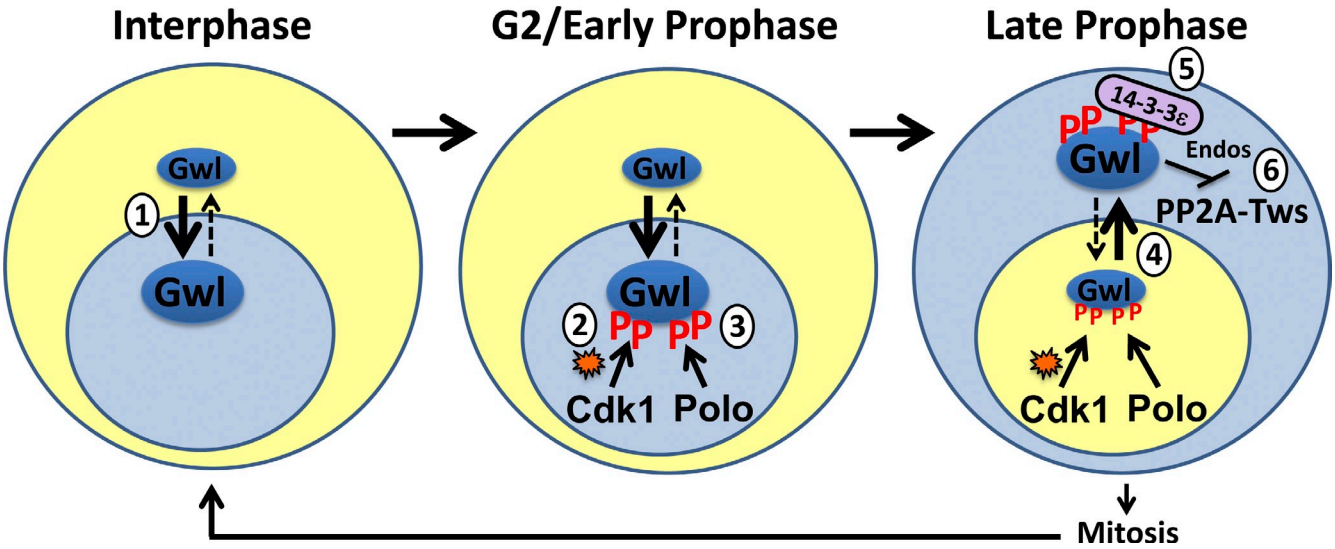


Figure 9. **An integrative model for the spatial regulation of Gwl.** Gwl is actively imported in the nucleus in interphase (1). In early prophase, cyclin B–Cdk1 activates Gwl in the nucleus (2). After phosphorylation of Gwl in its central region by Polo (3), and possibly by cyclin B–Cdk1 (2), Gwl then relocalizes to the cytoplasm before NEBD (4). Gwl is bound by 14-3-3ε, which helps its retention in the cytoplasm (5), where Gwl can inactivate PP2A-Tws to promote orderly mitotic entry (6). See text for details.

(Hara et al., 2012), which is consistent with the need to import cyclin B in the nucleus for Gwl activation. In agreement with this model, we find that cyclin B is imported into the nucleus at a time in G2 when Gwl is still in the nucleus (Fig. S5). Moreover, because PP2A-Tws/B55 is thought to dephosphorylate several cyclin B–Cdk1 substrates, it could potentially inactivate Gwl; therefore, the nuclear localization of Gwl could also facilitate Gwl activation by sequestering it away from PP2A-Tws/B55, which is known to be largely cytoplasmic in both *Drosophila* (Mayer-Jaekel et al., 1994) and human cells (Santos et al., 2012).

Activation of Gwl in the nucleus would be quickly followed by its translocation to the cytoplasm, which depends on its phosphorylation by Polo, followed by 14-3-3ε binding. Our results suggest that cyclin B–Cdk1 phosphorylation of Gwl in its central region is also required for Gwl's nuclear exclusion, although this phosphorylation is not required for Gwl's binding to Polo or 14-3-3. The requirement for multiple phosphorylation events dependent on two kinases (Polo and Cdk1) may ensure the robustness and proper timing of Gwl's relocalization in prophase, consistent with the physiological importance of this process. Previous studies have established that Gwl promotes mitotic entry by phosphorylating endosulfine proteins, which then become PP2A-Tws/B55 inhibitors (Gharbi-Ayachi et al., 2010; Mochida et al., 2010; Rangone et al., 2011). Endos is present in both the nucleus and the cytoplasm during the whole cell cycle (Rangone et al., 2011), and therefore, changes in Gwl localization likely dictate where Endos can be phosphorylated. Endos is a small protein that may diffuse freely through nuclear pores if not bound to other proteins, but its localization dynamics have not been studied. Phosphorylation of Endos by Gwl directly in the cytoplasm could help inhibit PP2A-Tws more efficiently in that compartment, and facilitate the accumulation of phosphorylated cyclin B–Cdk1 substrates and mitotic entry (Fig. 9).

Our results suggest that this spatial coordination of Gwl relative to other mitotic regulators is crucial for its function. The fact that the loss of Gwl function altogether does not prevent mitotic entry in many cell types but instead leads to mitotic defects (Yu et al., 2004; Archambault et al., 2007; Glover, 2012) already suggested that failure of the Gwl-PP2A axis in the mitotic switch impacts mitotic events after NEBD. The identity of the PP2A-B55 substrates that must be protected by Gwl from dephosphorylation at mitotic entry first needs to be discovered before we can dissect the importance of their regulation in space and time. These crucial substrates could be nuclear or cytoplasmic. Interestingly, we found that the nuclear retention of Gwl in prophase delays anaphase onset relative to NEBD (Fig. 6). This result suggests that PP2A-B55 must act on at least one nuclear substrate before NEBD. In yeast, it has recently been shown that PP2A-Cdc55 (B55) protects sister chromatid cohesion in early mitosis by antagonizing Polo phosphorylation of the Scc1 cohesin (Yaakov et al., 2012). A similar mechanism in animal cells could impose a need for Gwl's nuclear exclusion in late prophase. Although Tws (B55) is largely localized to the cytoplasm before NEBD, we have found that it shuttles in and out of the nucleus (unpublished data).

### A mechanism directly linking Gwl and Polo

We have shown that the spatial regulation of Gwl depends in part on the Polo kinase. Polo interacts with Gwl, phosphorylates it in its central region, and promotes its cytoplasmic localization (Figs. 4 and 5). Consistent with this mechanism, immunostaining in syncytial embryos reveals the presence of active pT182-Polo in prophase nuclei (Fig. S1 B). This is in agreement with a previous study in human cells using a FRET probe that showed that Plk1 activity appears in the nucleus in G2 and increases there during prophase (Macůrek et al., 2008). Moreover, Gwl-NLSmut (which does not enter the nucleus) appears less phosphorylated than Gwl-wt in Western blots (Fig. 3 E). NLSs are

often negatively regulated by phosphorylation in their proximity (Nardozzi et al., 2010). One classical example is the Cdk1-dependent phosphorylation and nuclear exclusion of the transcription factors Swi5 and Ace2 until mitotic exit in *Saccharomyces cerevisiae* (Moll et al., 1991). Phosphorylation of Gwl by Polo and Cdk1 could partially inactivate its NLSs by reducing the charge in their vicinity. In addition, binding of 14-3-3ε to phosphorylated Gwl could mask its NLSs, thereby preventing its nuclear import. A similar mechanism is known to operate to regulate the Cdk1-activating phosphatase CDC25C in human cells. Binding of 14-3-3 proteins to phosphorylated CDC25C mediates its retention in the cytoplasm until phosphorylation of CDC25C at other sites disrupts its interaction with 14-3-3 to allow the nuclear import of CDC25C that stimulates mitotic entry (Kumagai et al., 1998; Kumagai and Dunphy, 1999; Graves et al., 2001).

The current study shows a direct connection between Polo and Gwl and allows clearer interpretations of genetic results. By antagonizing Gwl's nuclear localization in interphase, increased Polo activity leaves less Gwl in the nucleus (Fig. 5), which would cause Gwl to be less efficiently activated by cyclin B–Cdk1 (Fig. 9). When *gwl* function is already abrogated, this is associated with lethality, with frequent failures of syncytial nuclei to enter mitosis as revealed by missing mitotic spindles, undercondensed chromosomes, and apparently intact nuclear envelopes (Fig. 8). In a previous report, we showed that the converse perturbations of the Gwl/Polo balance lead to a very different phenotype; females heterozygous for a gain-of-function allele of *gwl* ($gwl^{Scant}$) and for a *polo* null allele lay embryos that fail to hatch, and show a high incidence of mitotic defects with detached centrosomes in the syncytium. Overexpression of Gwl-wt genetically interacts with *polo* hypomorphs in the same way (Archambault et al., 2007). We subsequently showed that Gwl antagonizes PP2A-Tws in meiosis and mitosis, consistent with the model proposed from biochemical results in frog extracts (Gharbi-Ayachi et al., 2010; Mochida et al., 2010), and as expected, *polo* hypomorphic mutations strongly interacted with mutations abrogating PP2A-Tws (Wang et al., 2011). According to our model (Fig. 9), lowering Polo activity would lead to an increase in Gwl activity in the nucleus, resulting in the mitotic defects and death observed when Gwl activity is already increased (Archambault et al., 2007) or when PP2A-Tws activity is reduced (Wang et al., 2011). Consistent with this model, retention of Gwl in the nucleus in prophase by mutation of Polo consensus sites in the central region of Gwl leads to long mitotic delays, even in the presence of endogenous Gwl (Fig. 6). It remains possible that the genetic interactions between Polo, Gwl, and PP2A-Tws reflect multiple levels of functional interactions (Glover, 2012).

### A mode of regulation that could be generally conserved

Our results allow us to ascribe a first role to the central region of Gwl, which is to regulate its localization in the cell cycle. The large number of Polo phosphorylation sites in Gwl and the fact that they tend to be poorly conserved when considered individually (unpublished data) suggests that no single Polo phosphorylation site alone may be responsible for the regulation of Gwl's localization. Indeed, it has been proposed that many physiologically important multi-site phosphorylation events occur in protein segments whose general position and function is conserved, but whose precise amino acid sequence is not conserved (Serber and Ferrell, 2007; Holt et al., 2009). Although the central region of *Drosophila* Gwl that contains the NLSs is poorly conserved in sequence, its function in regulating localization in a phosphorylation-dependent manner could be conserved in Gwl orthologues, including MASTL in humans.

The yeast orthologue of Gwl, Rim15 is known to be regulated in its nucleo-cytoplasmic localization in response to phosphorylation (Pedruzzi et al., 2003). In this case, the TOR and Sch9 (PKB homologue) kinases promote the cytoplasmic localization of Rim15, but the molecular mechanisms have not been fully explored. Although Rim15 and its substrates Igo1/2 have been implicated in the regulation of mRNA metabolism in G0 (Talarek et al., 2010), and not in mitotic entry as for Gwl, the molecular mechanisms regulating Rim15 and Gwl could share general features.

The recent discovery of the Gwl-PP2A axis and of its major role in cell cycle regulation was brought by *Drosophila* genetics and biochemistry in *Xenopus* extracts, but the subcellular spatial dynamics of the pathway had been little explored. Here, we have shown that Gwl is spatially regulated in the cell cycle and that this aspect of its function is essential in vivo. Moreover, we have uncovered a molecular mechanism responsible for this regulation of Gwl that targets its central region, for which no function was previously known. Nucleo-cytoplasmic coordination of the main mitotic regulators including Gwl appears like a crucial level of cell cycle control.

## Materials and methods

#### Fly culture and transgenesis

Transgenic flies were made by BestGene Inc. by P-element–based insertions in the $w^{1118}$ background. *UASp-GWL-GFP* flies were published elsewhere (Archambault et al., 2007). The wild-type strain used was Oregon R. Viability and fertility tests in genetic rescue experiments were conducted as described previously (Archambault et al., 2007). In brief, for fertility tests, well-fed females in the presence of males were placed in tubes containing grape juice agar and allowed to lay eggs for 1 d before being removed. The percentage of hatched embryos was scored 24 h later. For viability tests, flies were crossed and the number of observed flies of the genotype of interest relative to their expected number in the progeny was expressed as a percentage.

#### DNA constructs

Most expression vectors were generated in the Gateway system (Invitrogen). Coding sequences were first cloned into the pDONR221λ entry vector. They were then recombined with destination vectors to generate the following expression plasmids: pMT-GFP-Gwl, pMT-Gwl-GFP, pAC5-Gwl-Myc, pAC5-Gwl-TAP, pAC5-PrA-GFP, pAC5-Gwl-PrA, pMT-$Polo^{T182D}$-GFP, pAC5-H2Av-mRFP, pUASp-Gwl-PrA, pUASp-Myc-Gwl, pUASp-Polo-Myc, pAC5-Polo-PrA, pAC5-PrA-14-3-3ε, and pAC5-PrA-14-3-3ζ, as well as all related truncations and mutants. The pDEST15-GST-Gwl vector was a gift of H. Rangone (University of Cambridge, Cambridge, UK). Point mutants were generated by QuikChange (Agilent Technologies) in entry clones or were synthesized by Bio Basic Inc.

#### Cell culture

All cells were in the D-Mel2 (D-Mel) background and were cultured in Express Five medium (Invitrogen). Stable cell lines expressing pMT-GFP-GWL, pMT-Gwl-GFP (wt and mutants) tagged proteins were generated as described

previously (Archambault et al., 2008). In brief, selection was based on resistance to 20 µg/ml blasticidin. Inducible pMT-based vectors already contained the resistance gene, whereas other vectors did not and were co-transfected with pCoBlast (Invitrogen).

**Immunofluorescence and Western blotting**

Antibodies used in immunofluorescence and Western blotting were anti–α-tubulin YL1/2 (Sigma-Aldrich), anti–γ-tubulin GTU88 (Sigma-Aldrich), anti-lamin and anti–cyclin B (both from the Developmental Studies Hybridoma Bank, Iowa City, IA), anti-Myc 9E10 (Santa Cruz Biotechnology, Inc.), anti-Polo MA294 (a gift from D. Glover, University of Cambridge, Cambridge, UK), anti-pT182-Polo (BioLegend), rabbit IgG (for PrA in TAP tag), and anti-Gwl (custom-made against full-length Gwl by Genscript). Immunofluorescence in embryos and in cells was done as described previously (Archambault et al., 2008). In brief, cells were fixed with 4% formaldehyde for 10 min, and permeabilized and blocked in PBS containing 0.1% Triton X-100 and 1% BSA (PBSTB). Cells were incubated with primary antibody diluted in PBSTB for 1 h to overnight, washed three times in PBSTB, and incubated with secondary antibodies for 1–2 h. Cells were washed several times in PBSTB and once in PBS before being mounted in Vectashield medium (Vector Laboratories) containing DAPI. 0–3-h-old embryos were dechorionated in 50% bleach before fixation using formaldehyde and methanol. Embryos were then rehydrated, permeabilized, blocked, and incubated with antibodies. DAPI was added in the secondary antibody solution and embryos were mounted in Vectashield.

**Microscopy**

Cells shown in Fig. 3 were acquired on a microscope (AxioImager; Carl Zeiss) with a 100× oil objective (NA 1.4 DICIII) and a camera (AxioCam HRm; Carl Zeiss), using AxioVision software (Carl Zeiss). All other images of fixed cells and embryos were acquired on a confocal microscope (510 Meta; Carl Zeiss) with a 100× oil objective (NA 1.4 DICIII), using LSM510 3.2 software (Carl Zeiss). Time-lapse imaging of embryos was done on a microscope (Eclipse Ti Swept-Field; Nikon) with a 60× oil objective (NA 1.1) and a camera (QuantEM:512SC; Photometrics), using NIS Elements software (Nikon). Time-lapse imaging of GFP-Gwl– or Gwl-GFP–expressing cells was performed using a spinning disc confocal system (Ultraview Vox; PerkinElmer), a PlanApo 100× oil immersion objective (NA 1.4), and a CCD camera (Orca-R2; Hamamatsu Photonics) with 2 × 2 binning and using Volocity 6.0 software (Improvision/PerkinElmer; Videos 1–5, Fig. 6). Other time-lapse images were acquired on a DeltaVision microscope with a 60× oil objective (NA 1.42) and a camera (CoolSNAP HQ$^2$; Photometrics) with binning 2 × 2, at 25°C using SoftWoRx 5.5 Explorer (Applied Precision; Fig. 5 D). The quantifications in Figs. 5 B and 7 D were done using ImageJ (National Institutes of Health). All images were acquired at room temperature unless otherwise specified.

**Affinity purifications**

Protein A affinity purifications from cells were performed essentially as described previously (D'Avino et al., 2009). In brief, pelleted cells from confluent 75-cm$^2$ flasks were resuspended in ∼10 volumes of lysis buffer and passed through a needle several times using a syringe. Lysates were clarified by centrifugation for 15 min at 14,000 rpm in a tabletop centrifuge. Supernatants were incubated with 25 µl of IgG-conjugated DynaBeads (Invitrogen) for 1–2 h and washed with lysis buffer 4–5 times for 5 min. Purification products were eluted by heating at 95°C for 2 min in SDS-PAGE Laemmli buffer (Sigma-Aldrich) and analyzed by Western blotting. Purifications from embryos were performed following very similar protocol. 0–3-h-old embryos were crushed thoroughly in lysis buffer and centrifugated for 15 min at 14,000 rpm in a tabletop centrifuge. Clarified supernatants were taken, without disturbing the fatty layer on top. The centrifugation was repeated and any residual fatty fraction was discarded. The remaining steps of the purifications were as with cells.

**Kinase assays**

Active HIS-Polo obtained from Sf9 cells was used to phosphorylate GST-Gwl obtained from bacterial expression. Cyclin B–Cdk1 (human) was from EMD Millipore. Reactions were done in kinase buffer (20 mM K-Hepes, 2 mM $MgCl_2$, 1 mM DTT, 1 µM ATP, and 1 µCi γ-[$^{32}$P]ATP) at 30°C for 20 min and stopped with the addition of Laemmli buffer. Products were resolved by SDS-PAGE and transferred onto nitrocellulose for autoradiography.

**Phosphorylation site mapping**

Samples were reconstituted in 50 mM ammonium bicarbonate with 10 mM TCEP and vortexed for 1 h at 37°C. Chloroacetamide was added for alkylation to a final concentration of 55 mM. Samples were vortexed for another hour at 37°C. 1 µg of trypsin was added and the digestion was performed for 8 h at 37°C. Samples were dried down and solubilized in ACN 5% formic acid (FA) 0.2%. Samples were loaded on a homemade C18 precolumn (0.3 mm i.d. x 5 mm) connected directly to the switching valve and separated on a homemade reversed-phase column (150 µm i.d. x 150 mm) with a 56-min gradient from 10–60% acetonitrile (0.2% FA) and a 600 nl/min flow rate on a NanoLC-2D system (Eksigent) connected to an LTQ-Orbitrap Elite (Thermo Fisher Scientific). Each full MS spectrum acquired with a 60,000 resolution was followed by 12 MS/MS spectra, where the 12 most abundant multiply charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using the Mascot 2.4 (Matrix Science) and the Uniprot *Drosophila melanogaster* database (58,894 sequences). Tolerances on precursors and fragments were 15 ppm and 0.5 D, respectively. Variable selected post-translational modifications were carbamidomethyl (C), oxidation (M), deamidation (NQ), and phosphorylation (STY).

**RNA interference**

dsRNAs corresponding to the *GWL* coding sequence between bp 200 and 625 or to the bacterial kanamycin resistance gene (as control) were synthesized after PCR using T7 sequence-containing primers using the T7 Ribomax kit (Promega). To obtain RNAi-resistant forms of Gwl-GFP, we replaced all codons in the region targeted by the GWL dsRNA with alternative, conservative codons. DNA cassettes were synthesized by Bio Basic Inc. and were subcloned into pDONR-GWL (wt and NLSmut), allowing the generation of pMT-Gwl$^{RES}$-GFP and pMT-Gwl-NLSmut$^{RES}$-GFP, for copper-inducible expression. Stable cell lines were generated with these constructions. The day before RNAi treatment, 500,000 cells were plated in 1 ml of medium in 12-well plates. RNAi was induced with 15 µg of dsRNA transfected with Transfast (Promega). Cells were analyzed after 4 d.

**Preparation of GST-fusion proteins**

Overnight cultures of BL21 *Escherichia coli* transformed with pGEX-14-3-3ε or 14-3-3ζ, or corresponding mutants (K49Eε or K51Eζ), were diluted into 500 ml and expression induced with 1 mM IPTG overnight at 25°C. Cells were pelleted and resuspended in 40 ml of PBS, 0.1 M EDTA, and 0.1% Triton-X, and supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride), 5 µg/ml pepstatin A, 10 µg/ml leupeptin, and 1 mM PMSF. Bacterial cell suspensions were lysed by sonication and extracts were centrifugated at 13,000 *g* for 30 min, and were stored in 1-ml aliquots at −80°C until they were used. For preparation of beads, 1 ml of clarified extract was incubated with 200 µl of washed 50% slurry of glutathione–Agarose (G-Biosciences) for 8 h. Beads were washed four times with PBS, 0.1 M EDTA, 0.1% Triton-X, and then three times with cell lysis buffer (see below). Before use, GST-bound 14-3-3 protein was normalized and quantified using Coomassie staining of BSA as a standard. All experiments were performed at 4°C.

**GST pull-down assay**

For pull-down assays, D-Mel cells were washed with ice-cold PBS and lysed in 10 mM $K_3PO_4$, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% NP-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate ($Na_3VO_4$), 1 mM PMSF, and Complete protease inhibitors (Roche). Clarified lysates were incubated with 10 µg of GST-bound 14-3-3 proteins for 2 h, after which beads were washed three times with cell lysis buffer before SDS-PAGE and immunoblotting.

**Online supplemental material**

Fig. S1 shows that the localization of Gwl is cell cycle regulated in embryos and is a complement to Fig. 1. It also shows that active Polo is nuclear in prophase. Fig. S2 shows a sequence alignment between Gwl orthologues from *Drosophila* and mosquito species, where the NLS motifs are conserved. Fig. S3 documents the phosphorylation site mapping in Gwl. Fig. S4 shows that Polo phosphorylation of Gwl promotes its cytoplasmic localization as a complement to Fig. 5. Fig. S5 shows that cyclin B is imported in the nucleus while Gwl is still in the nucleus. Videos 1–5 show time-lapse imaging of mitosis in a D-Mel cell expressing GFP-Gwl (Video 1), GFP-SV40NLS-Gwl (Video 2), Gwl-GFP (Video 3), Gwl-PoloA-GFP (Video 4), and Gwl-CdkA-GFP (Video 5). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201211141/DC1.

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