SUPPLEMENTARY INFORMATION

Supplementary Information includes 5 figures, 19 Movies, Supplemental Material and Methods and Supplementary References

Supplemental figures and legends

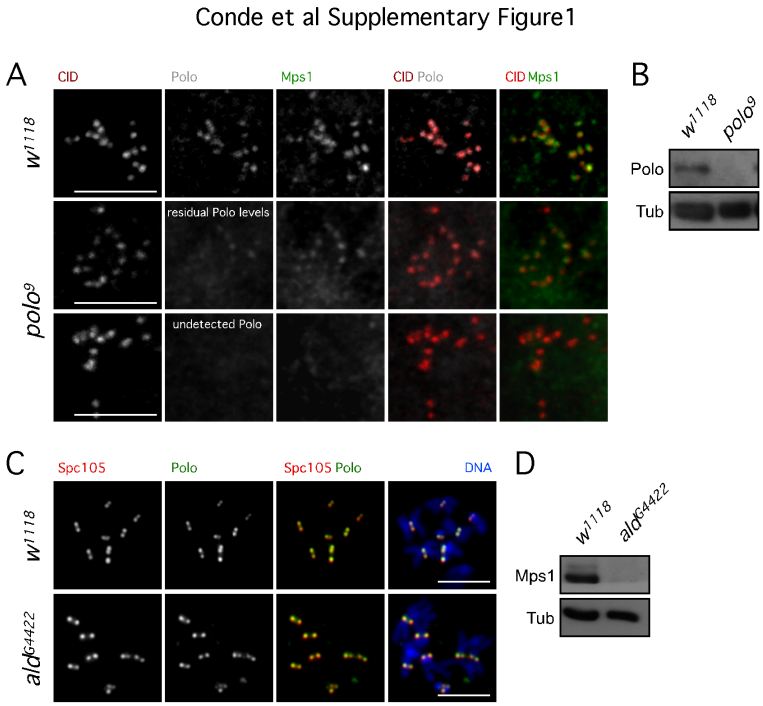

Figure S1. Polo is required for Mps1 kinetochore localization in neuroblasts.

(A) Kinetochore localization of Mps1 and Polo in 3rd *instar* larvae neuroblasts from w^{1118} and hypomorph $polo^{9}$ homozygous. CID staining was used as a kinetochore reference. Due to the hypomorphic nature of $polo^{9}$ allele, residual levels of Polo could be detected at kinetochores of some neuroblasts. (B) Immunoblot analysis confirming the dramatic reduction of Polo levels in $polo^{9}$ homozygous larvae. (C) Kinetochore localization of Polo in 3rd *instar* larvae neuroblasts from w^{1118} and ald^{G4422} homozygous. (D) Immunoblot confirming the absence of detectable Mps1 in ald^{G4422} homozygous larvae. Scale bars represent 5 μm.

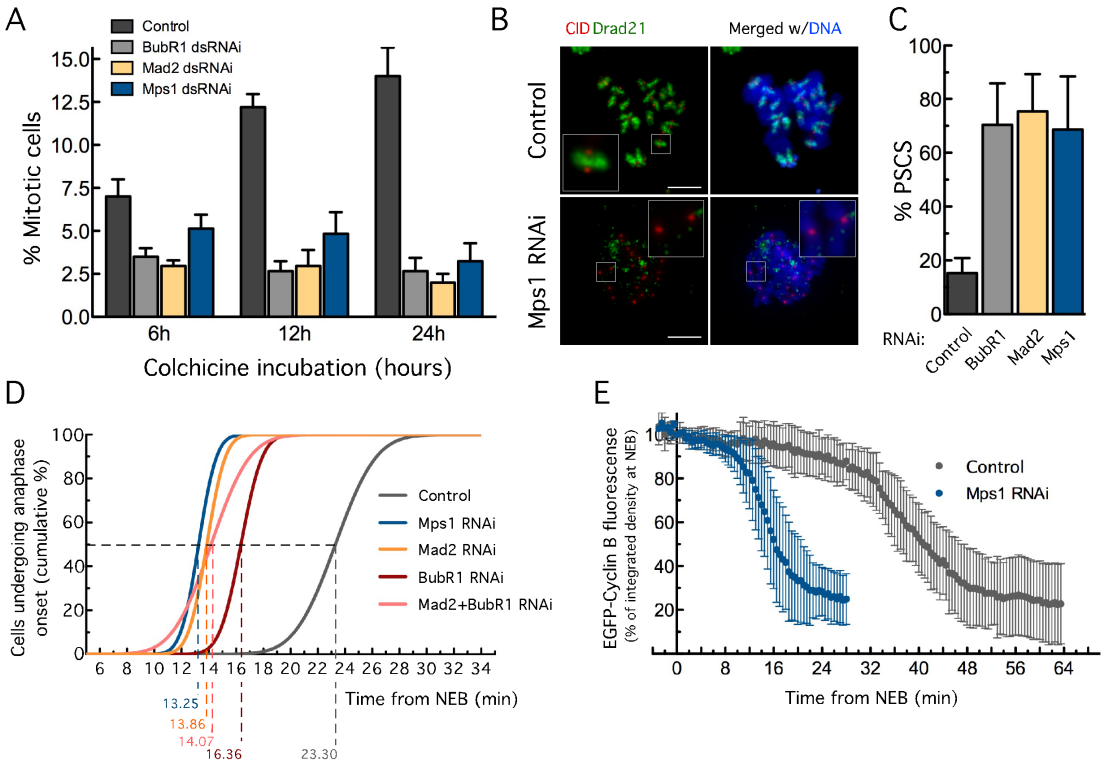

Figure S2. Analysis of SAC function and mitotic timing in S2 cells depleted of Mps1

(A) Mitotic index quantification based on $H3^{Ser10Ph}$ staining. Data represents mean ± SD, n > 3000 cells for each condition, from three independent experiments. (B) Immunofluorescence analysis of Drad21, to assess precocious sister chromatid separation (PSCS). CID staining was used as a kinetochore reference. Insets show higher-magnification views of boxed areas. (C) Quantification of PSCS. Data are mean ± SD, n > 300 mitotic cells for each condition. (D) Quantification of mitotic timing in control and RNAi-treated cells stably expressing mCherry-CID and EGFP-Tubulin. The depicted lines represent cumulative percentages of cells that undergo anaphase onset at indicated times after NEB. Dashed lines indicate the mean time for each experimental condition. (E) Cyclin B degradation profiles in cells expressing

EGFP-Cyclin B and mCherry-Tubulin. Data represent the mean ± SD of at least 10 cells for each experimental condition. EGFP-Cyclin B fluorescence at NEB was set to 100%. Scale bars represent 5 μm.

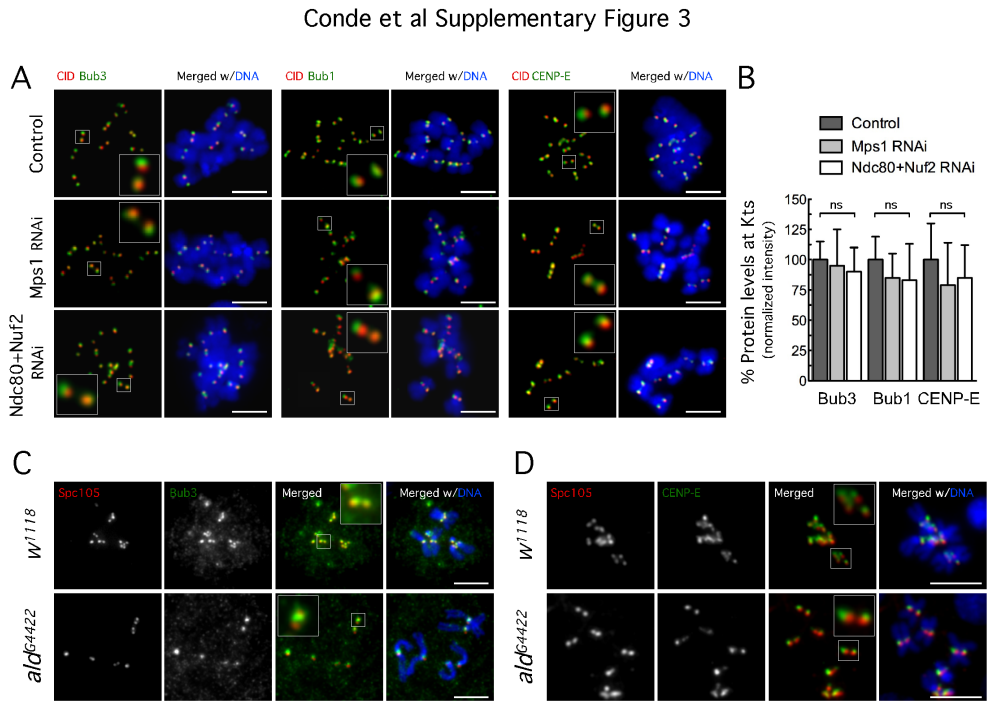

Figure S3. Mps1 is not required for kinetochore localization of Bub1, Bub3, or CENP-E.

(A) Kinetochore localization of Bub3, Bub1 and CENP-E in S2 cells for each of the indicated conditions. (B) Quantification of experiment in (A). Signal intensities at kinetochores were determined using CID signals as reference. Data represent mean ± SD, One-way ANOVA Turkey's multiple comparison test. n > 20 cells for each condition. Cells were treated with MG132 for 1 hour followed by 2 hours of colchicine incubation. Mean values obtained for control cells were set to 100%. (C-D) Immunofluorescence images of 3rd *instar* larvae neuroblasts from w^{1118} and ald^{G4422} homozygous stained for Bub3 (C) and CENP-E (D). Spc105 was used as kinetochore

reference. Insets show higher-magnification views of selected kinetochore pairs. Scale bars represent 5 μm.

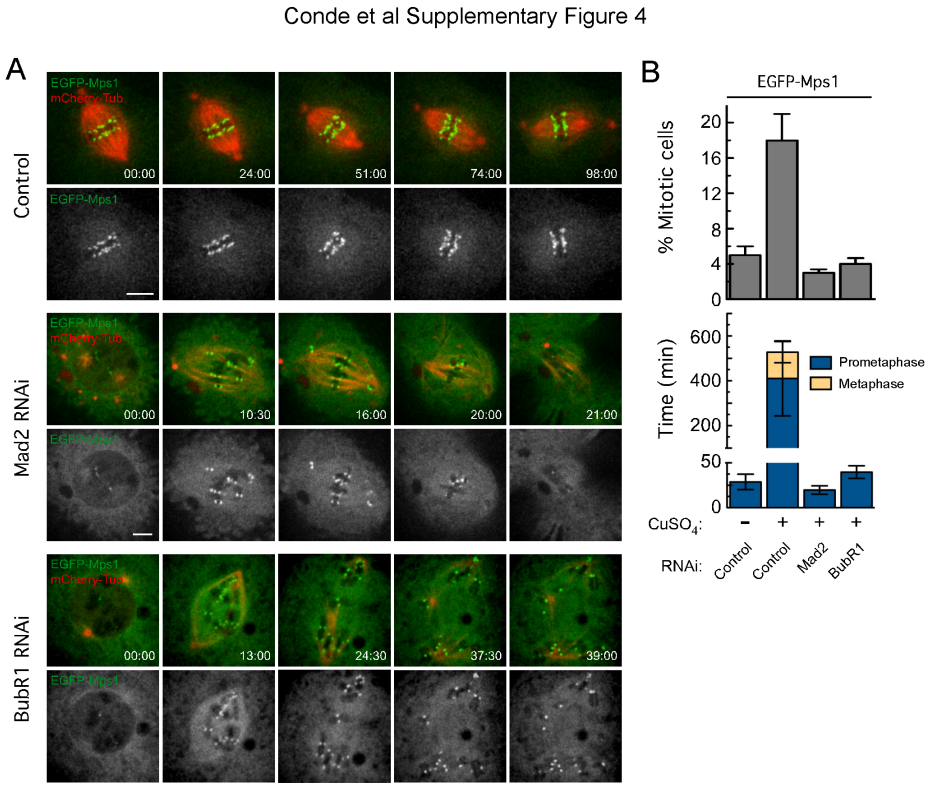

Figure S4 Mps1 overexpression causes a prolonged, SAC-dependent, mitotic arrest.

(A) Mitotic progression of control and dsRNAi treated cells co-transfected with mCherry-Tubulin and EGFP-Mps1 was monitored by time-lapse microscopy. Selected stills from 4D microscopy analysis of control and RNAi treated cells are shown. Time is in min:sec. EGFP-Mps1 overexpression was induced with 50 μM CuSO4 for 12 hours. (B) Graphs represent mitotic indices and mitotic timing of experiments in (A). Values in bar graphs represent mean ± SD. For mitotic index determination at least 3000 cells of each condition were considered. For quantification of mitotic timing at least 10 cells of each condition were monitored. Scale bars correspond to 5 μm.

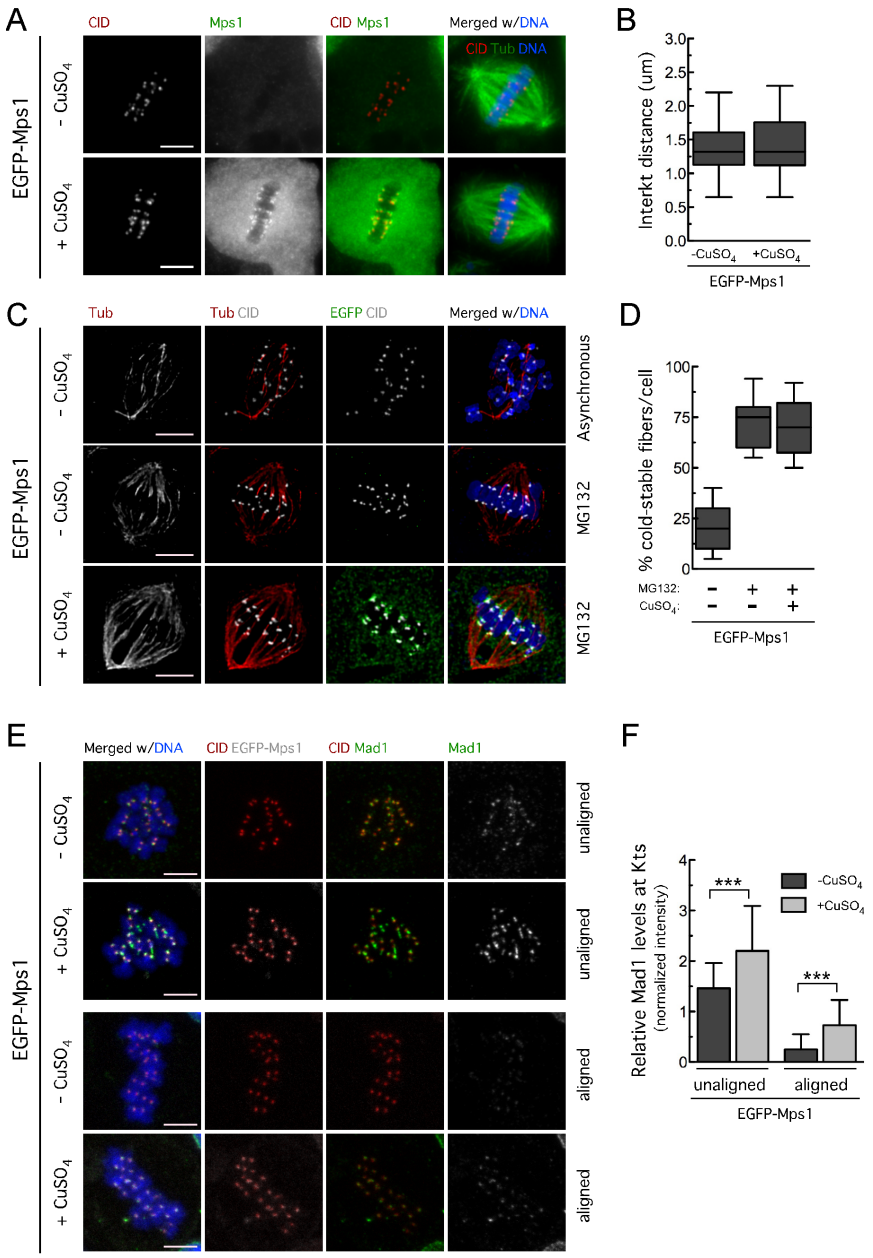

Figure S5. Overexpressed Mps1 is able to localize at microtubule-attached kinetochores.

(A) Immunofluorescence analysis of metaphase cells exclusively expressing endogenous Mps1 (-$CuSO_4$) and overexpressing EGFP-Mps1 (+$CuSO_4$) reveals that under overexpression conditions, Mps1 is able to localize at kinetochores of aligned chromosome that are under tension. CID immunolocalization was used as kinetochore

reference. (B) Box and whiskers plots of interkinetochore distances measured in aligned chromosomes of experiment in (A). n > 15 cells for each condition. (C) Immunofluorescence analysis of stable kinetochore-fibers and Mps1 immunolocalization after cold treatment in the indicated experimental conditions. (D) Box and whiskers plots of the quantification of the % of cold-fibers/cell from experiment in (C). n > 15 cells for each condition. (E) Mad1 and Mps1 kinetochore localization in metaphase chromosomes of non-induced (-$CuSO_4$) and in overexpressing EGFP-Mps1 cells (+$CuSO_4$) and respective quantification. CID signal was used as kinetochore reference, relative to which Mad1 fluorescence intensity at kinetochores was determined. Data represents mean ± SD and the mean values obtained for control cells were set to 100%. ***p < 0.001, Student *t* test test. n >10 cells for each condition. Scale bars correspond to 5 μm.

Supplemental Movies

Movie S1. Cyclin B degradation pattern in S2 control cells. (Related to Figure 1B, C and Figure S2E). Mitotic progression of S2 cells co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) was followed by spinning disk confocal microscopy. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S2. Cyclin B degradation pattern in S2 cells co-depleted of Mad2 and BubR1. (Related to Figure 1B, C). Mitotic progression of S2 cells co-depleted of Mad2 and BubR1 co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) was followed by spinning disk confocal microscopy. Video illustrates

premature degradation of EGFP-Cyclin B. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S3. Cyclin B degradation pattern in S2 cells depleted of Polo. (Related to Figure 1B, C). Mitotic progression of S2 cells depleted of Polo co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) was followed by spinning disk confocal microscopy. Video illustrates the prolonged mitotic arrest with high levels of Cyclin B resulting from Polo depletion. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 2 minutes. The first frame corresponds to time 0:0. Time is shown minutes.

Movie S4. Cyclin B degradation pattern in S2 cells depleted of Polo, Mad2 and BubR1. (Related to Figure 1B, C). S2 cells depleted of Polo, Mad2 and BubR1 co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) were followed by spinning disk confocal microscopy. Video illustrates the prolonged mitotic arrest with high levels of Cyclin B resulting from loss of Polo. The inability of Mad2 and BubR1 co-depletion to override the arrest indicates that it is SAC-independent. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 2 minutes. The first frame corresponds to time 0:0. Time is shown minutes.

Movie S5. Cyclin B degradation pattern in Polo-inhibited S2 cells. (Related to Figure 1B, C). S2 cells co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) were incubated with Polo inhibitor BI2536 for 2 hours and followed by

spinning disk confocal microscopy. Video illustrates the prolonged mitotic arrest with high levels of Cyclin B resulting from loss of Polo activity. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 2 minutes. The first frame corresponds to time 0:0. Time is shown minutes.

Movie S6. Cyclin B degradation pattern in Polo-inhibited S2 cells co-depleted of Mad2 and BubR1. (Related to Figure 1B, C). S2 cells co-depleted of Mad2 and BubR1 co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) were incubated with Polo inhibitor BI2536 for 2 hours and followed by spinning disk confocal microscopy. Video illustrates the prolonged mitotic arrest with high levels of Cyclin B resulting from loss of Polo activity. The inability of Mad2 and BubR1 co-depletion to override the arrest indicates that it is SAC-independent. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 2 minutes. The first frame corresponds to time 0:0. Time is shown minutes.

Movie S7. Mitosis in EGFP-Polo^{WT} expressing cells. (Related to Figure 3G, H). Mitotic progression of S2 cells expressing EGFP-Polo^{WT} was followed by spinning disk confocal microscopy. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S8. Expression of EGFP-Polo^{T182D} causes a metaphase delay (Related to Figure 3G, H). Mitotic progression of S2 cells expressing EGFP-Polo^{T182D} was followed by spinning disk confocal microscopy. Video illustrates the metaphase delay

resulting from EGFP-Polo^{T182D} expression. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S9. Depletion of Ndc80 and Nuf2 prevents the metaphase delay caused by EGFP-Polo^{T182D} expression. (Related to Figure 3G, H). Mitotic progression of S2 cells expressing EGFP-Polo^{T182D} and co-depleted of Ndc80 and Nuf2 was followed by spinning disk confocal microscopy. Video illustrates how the mitotic delay resulting from EGFP-Polo^{T182D} expression is abrogated by depletion of Ndc80 and Nuf2. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S10. Mitotic timing in control S2 cells. (Related to Figure S2D). Mitotic progression of S2 cells expressing mCherry-CID (red) and GFP-α-Tubulin (green). mCherry-CID signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S11. Mps1 depletion results in premature anaphase onset. (Related to Figure S2D). Mitotic progression of Mps1-depleted S2 cells expressing mCherry-CID (red) and GFP-α-Tubulin (green). mCherry-CID signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S12. Mps1 depletion results in premature Cyclin B degradation (Related to Figure S2E). Mitotic progression of Mps1-depleted S2 cells co-expressing mCherry-α-Tubulin (red) and EGFP-Cyclin B (green) was followed by spinning disk confocal microscopy. Video illustrates premature degradation of EGFP-Cyclin B upon Mps1 depletion. EGFP-Cyclin B signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S13. *In vivo* analysis of BubR1 localization pattern in w^{1118} neuroblasts. (Related to Figure 4D). Mitotic progression of w^{1118} neuroblasts expressing mRFP-α-Tubulin (red) and BubR1-GFP (green) was followed by spinning disk confocal microscopy. BubR1-GFP signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S14. *In vivo* analysis of BubR1 localization pattern in ald^{G4422} mutant neuroblasts. (Related to Figure 4D). Mitotic progression of ald^{G4422} neuroblasts expressing mRFP-α-Tubulin (red) and BubR1-GFP (green) was followed by spinning disk confocal microscopy. Video illustrates that Mps1 is dispensable for BubR1 kinetochore localization in neuroblasts mitosis. BubR1-GFP signal alone is shown on the right. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S15. *In vivo* analysis of BubR1 localization pattern in S2 control cells. Mitotic progression of S2 cells co-expressing GFP-α-Tubulin (green) and mRFP-

BubR1 (red) was followed by spinning disk confocal microscopy. mRFP-BubR1 signal alone is shown on the bottom. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S16. *In vivo* analysis of BubR1 localization pattern in Mps1-depleted S2 cells. Mitotic progression of Mps1-depleted S2 cells co-expressing GFP-α-Tubulin (green) and mRFP-BubR1 (red) was followed by spinning disk confocal microscopy. Video illustrates that Mps1 is dispensable for BubR1 kinetochore localization in S2 mitosis. mRFP-BubR1 signal alone is shown on the bottom. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S17. Mps1 overexpression causes a pronounced metaphase arrest. (Related to Figure S4A, B). S2 cells overexpressing EGFP-Mps1 (green) and mCherry-α-Tubulin (red) were followed by spinning disk confocal microscopy. Video illustrates the prolonged metaphase arrest with EGFP-Mps1 present at kinetochores of bi-oriented chromosomes. EGFP-Mps1 signal alone is shown on the bottom. Each frame represents a maximal intensity projection acquired every 60 seconds. Time 0:0 corresponds to the first metaphase frame. Time is shown minutes.

Movie S18. Mad2 depletion abrogates the mitotic arrest caused by Mps1 overexpression. (Related to Figure S4A, B). Mitotic progression of Mad2-depleted cells overexpressing EGFP-Mps1 (green) and mCherry-α-Tubulin (red) was followed by spinning disk confocal microscopy. Video illustrates that Mad2 depletion results in

premature mitotic exit indicating that the mitotic arrest caused by Mps1 overexpression is due to SAC activity. EGFP-Mps1 signal alone is shown on the bottom. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Movie S19. BubR1 depletion abrogates the mitotic arrest caused by Mps1 overexpression. (Related to Figure S4A, B). Mitotic progression of BubR1-depleted cells overexpressing EGFP-Mps1 (green) and mCherry-α-Tubulin (red) was followed by spinning disk confocal microscopy. Video illustrates that BuR1 depletion results in premature mitotic exit indicating that the mitotic arrest caused by Mps1 overexpression is due to SAC activity. EGFP-Mps1 signal alone is shown on the bottom. Each frame represents a maximal intensity projection acquired every 30 seconds. NEB corresponds to time 0:0. Time is shown minutes.

Supplemental Material and Methods

Fly stocks

Analysis of Polo kinase-dependent protein localization was performed on a hypomorphic mutant allele, *polo*^{9}, characterized by a P-element insertion that results in expression of low levels of Polo (Donaldson *et al*, 2001). For analysis of Mps1 mutant neuroblasts, a strain containing a P-element insertion within the Mps1/ald gene (*ald*^{G4422}) impairing Mps1 expression was used. Both stocks were obtained from Bloomington Stock Centre, Indiana, USA. Flies expressing BubR1-GFP under regulation of BubR1 natural promoter were kindly provided by R. Karess (Buffin *et al*, 2005). *w1118* was used as a control strain.

S2 cell cultures, dsRNAi and drug treatments

Drosophila S2 cell cultures, dsRNAi synthesis and dsRNAi treatments were performed according Maiato et al. (Maiato *et al*, 2003). At selected time points, cells were collected and processed for immunofluorescence, time-lapse microscopy, immunoprecipitation or immunoblotting. When required, cells were subjected to several drug treatments before being collected and processed for the desired analysis. In order to promote microtubule depolymerization, cells were incubated with 30 μM colchicine (Sigma) for selected time periods. To prevent mitotic exit in a checkpoint independent manner, cells were incubated for 1-3 hours with proteasome inhibitor MG132 (Calbiochem) at final concentration of 20 μM. To inhibit Aurora B and Polo activities, 20 μM Binucleine 2 (Sigma) and 100 nM BI2536 (Boehringer Ingelheim) were respectively added to cultured cells prior microtubule depolymerization.

Constructs and cell transfection

To generate a cell line expressing constitutively active Polo-EGFP, a 300bp DNA fragment containing the mutation for Polo^{T182D} was generated by gene synthesis from GeneArt® Life technology (Invitrogen) and replaced the corresponding region of wild-type Polo cDNA previously cloned in the pGEMTeasy vector. Polo full coding region was then amplified by PCR with specific primers suitable for Gateway cloning system (Invitrogen). Expression vector pWGPolo^{T182D}-EGFP was generated by Gateway recombination (Invitrogen) of pENTR-Polo^{T182D} clone into pH-WG (heat-shock inducible promoter, C-terminal EGFP tag) destination vector. The same strategy was used to obtain pWGPolo^{WT}-EGFP and pGW-BubR1-EGFP expression vector. To generate the cell line expressing EGFP-Mps1 and EGFP-Cyclin B, Mps1 and Cyclin B coding regions was cloned in frame with EGFP under regulation of a

metallothionein promoter in the pMT vector (Invitrogen). Stable transfection of recombinant plasmids into S2 cells was performed using Cellfectin® II reagent, Life technology (Invitrogen), according to the manufacturer's instructions. To induce $Polo^{T182D}$-EGFP, $Polo^{WT}$-EGFP and BubR1-EFGP expression, cells were incubated for 30 min at 37 ºC 6 hours before being processed for immunofluorescence or immunoblotting. To induce EGFP-Mps1 and EGFP-Cyclin B expression, CuSO4 was added to cultures at a final concentration of 50 μM for 12 hours before analysis.

Immunoprecipitation and Immunoblotting

For immunoprecipitation experiments, S2 cultured cells were treated with MG132 for 1 hour followed by incubation with colchicine for 2 hours. Cells were harvested through centrifugation at 5000 rpm for 10 min at 4°C and afterwards washed with 2 mL PBS supplemented with protease inhibitors cocktail (Roche). Cell pellet was resuspended in lysis buffer (150 mM KCl, 7.5 mM Hepes, pH 7.5, 1.5 mM EGTA, 1.5 mM $MgCl_2$, 15% glycerol, 0.1% NP-40, 1× protease inhibitors cocktail (Roche) and 1× phosphatase inhibitors cocktail 3 (Sigma)) before disruption through freezing in liquid nitrogen. Cell lysates were then clarified through centrifugation at 8 000 rpm for 10 min at 4°C and quantified by Bradford protein assay (Bio-Rad). 2 mg of extract were used for each immunoprecipitation assay. Lysates were pre-cleared through incubation with 20 µL of magnetic bead bound Protein A or G (New England Biolabs) for 1h at 4°C with rotation, in order to reduce non-specific binding. Pre-cleared extracts were incubated at 4ºC overnight with the primary antibodies. Afterwards they were incubated with 50 µL of magnetic bound Protein A or G for 1,5h at 4°C with rotation. The magnetic beads and bound protein fraction were collected and washed 5 times with 1 mL of lysis buffer. Finally the magnetic beads

and bound protein were resuspended in 20 µL of 2x laemmli buffer (4% SDS, 10%Mercaptoethanol; 125 mM Tris-HCl; 20% Glycerol, 0,004% Bromophenol blue and boiled for 5 min at 95ºC. After removal of the magnetic beads, samples were resolved by SDS-PAGE and probed for proteins of interest through western blotting. For Western blot analysis, resolved proteins were transferred to a nitrocellulose membrane, using the iBlot Dry Blotting System (Invitrogen) according to the manufacturer's instructions. Transferred proteins were confirmed by ponceau staining (0.25% Ponceau S in 40% methanol and 15% acetic acid). The membrane was blocked for 3 hour at room temperature with 5% dry milk in PBS-T. All the primary and secondary antibodies were diluted in PBS-T containing 1% BSA and the membranes were incubated overnight at 4ºC under agitation, then washed three times 10 min with PBS-T and immediately incubated with secondary antibodies diluted in 1% dry milk in PBS-T 1 hour at room temperature under agitation. Anti-rabbit, anti-mouse and anti-guinea pig secondary antibodies conjugated to Horseradish peroxidase (Amersham) were used according to the manufacturer´s instructions. Blots were developed with ECL Chemiluminescent Detection System (Amersham) according to manufacturer's protocol and detected on X-ray film (Fuji Medical X-Ray Film). To confirm protein hyperphosphorylation status, 50 μg of control cell lysates were treated with 20 U of alkaline phosphatase (Fermentas, FastAP) for 1 hour at 37˚C. A GS800 densitometer and Quantity One software (Bio-Rad) were used for quantitative immunoblotting when required.

Antibodies

The primary antibodies used were anti-α-tubulin mouse B512 (Sigma) used at 1:4000 for immunofluorescence (IF), anti-α-tubulin mouse DM1A (Sigma) used at

1:10000 for immunoblotting (IB), anti-phosphorylated ser10-Histone H3 rabbit (Upstate Biotechnology) used at 1:250 (IB) and 1:10000 (IF), anti-BubR1 (Rb 666) rabbit used at 1:3000 (IF), anti-Mad1 rabbit used at 1:2000 (IF), 1:50 for immunprecipitation (IP) and 1:3500 (IB), anti-Bub1 (Rb 1112) rabbit used at 1:100 (IF), anti-Bub3 (Rb 730) rabbit used at 1:10 (IF) and 1:500 (WB), anti-CID rat used at 1:1500 (IF), anti-Fizzy/Cdc20 rabbit (gift from Jordan Raff) used at 1:100 (IF), 1:1000 (IB) and 1:50 (IP), anti-Cdc27 rabbit (gift from Jordan Raff) used at 1:1000 (IB), anti-Mps1 (Gp15) guinea pig (gift from Scott Hawley) used at 1:250 (IF), 1:100 (IP) and 1:3000 (IB), anti-Scc1/Drad21 guinea pig (gift from S. Heidman) used at 1:2000 (IF), anti-Mad2 (Rb 1223) rabbit used at 1:100 (IB), 1:50 (IP) and 1:10 (IF), anti-Aurora B (Rb2) rabbit (gift from D. Glover) used at 1:50 (IF), anti-CENP-E (Rb205) rabbit (gift from Byron Williams) used at 1:1000 (IF), anti-Ndc80 (Rb272) rabbit (gift from M. Goldberg) used at 1:5000 (IB), anti-Cyclin B rabbit (gift from C. Lehner) used at 1:25000 (IB), anti-Polo (MA294) mouse used at 1:30 (IF) and 1:50 (IB), anti-Mad1 rabbit used at 1:2000 (IF, IB), anti-Zw10 (Rb83) rabbit (gift from M. Goldberg) used at 1:2000 (IB) and 1:1000 (IF), anti-Spc105 used at 1:200 (IF), anti-EGFP used at 1:50 (IP) and 1:250 (IF), anti-PhosphoT210 Plk1 (ab39068) mouse (Abcam) used at 1:10000 (IF) and anti-3F3/2 mouse (gift from G. Gorbsky) used at 1:500 (IF), anti-FK2 mouse (Enzo Life Sciences) used at 1:250 (IB). Secondary antibodies with conjugated HRP (Santa Cruz) or fluorescent dyes from the Alexa series (Invitrogen) were used according to manufacturer's instructions.

Supplementary references

Buffin E, Lefebvre C, Huang J, Gagou ME and Karess RE (2005) Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. *Curr Biol* **15:** 856–861

Donaldson MM, Tavares AA, Ohkura H, Deak P and Glover DM (2001) Metaphase

arrest with centromere separation in polo mutants of Drosophila. *J Cell Biol* **153:** 663–676

Maiato H, Sunkel CE and Earnshaw WC (2003) Dissecting mitosis by RNAi in Drosophila tissue culture cells. *Biol Proced Online* **5:** 153–161