Supplemental Data

Drosophila Wee1 Kinase Regulates Cdk1 and Mitotic Entry during Embryogenesis

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Supplemental Experimental Procedures

Stocks

Wild-types are Sevelin or *yw* (for phospho-Cdk1 staining only). Others have been described: *dwee1*^{ES1} and Df(2L)*dwee1*^{W05} [S1]; *mnk*^{6006} (DmChk2) [S2]; and 17238-GFP and Histone-GFP transgenic stocks [S3, S4]. Fly stocks carrying *dwee1* and GFP transgenes or *dwee1, mnk* double mutants were made by standard *Drosophila* genetics.

GST-Dwee1 Purification

dwee1 cDNA was cloned by RT-PCR with total RNA from S2 cells as template. The *dwee1* open reading frame (ORF) was subcloned into pDONR221 with Gateway technology (Invitrogen) and subsequently shuttled into pDEST.GST. GST-Dwee1 was induced in *E. coli* and purified by glutathione Sepharose affinity chromatography according to the manufacturer's instructions (Amersham-Pharmacia Biotech).

Kinase Assays

H1 kinase assays were performed on extracts from 1–2 hr-old embryos as previously described [S5], with the following modifications: 4 μl of rabbit anti-cyclin B antibody was used for immunoprecipitation from 50 μg of total protein (measured by Bradford assay; Pierce) in 250 μl EB. The reactions were incubated for 30 min at 25°C. SDS-PAGE gels were transferred to PVDF membrane (Immobilon). Blots were stained with Ponceau-S (Sigma). ^{32}P incorporation was detected by a Phosphorimager (Molecular Dynamics). ^{32}P signal was quantified by liquid scintillation counting of excised, Ponceau-S-stained H1 bands. For Western blots, antibodies were used at the following dilutions: mouse anti-PSTAIR (Cdk1), 1:1000 (Sigma); mouse anti-β-tubulin, 1:500; rabbit anti-GST, 1:1000 (Cell Signaling); donkey anti-mouse-HRP, 1:5000; and donkey anti-rabbit-HRP, 1:10000 (Amersham).

For GST-dWee1 inhibition assays, immunoprecipitations were performed as described above before 10 μl dWee1 kinase buffer (50 mM HEPES [pH 7.6], 1 mM EGTA, 15 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM $NaVO_4$, 1 μM microcystin, and 300 μM ATP) and 300 ng GST-dWee1 were added. Reactions were incubated for 30 min at 25°C before addition of 250 μg/ml histone H1 and 0.5 mCi/ml [γ-^{32}P] ATP. Reactions were incubated for another 30 min before being processed as above.

Phospho-Cdk1 Staining

For Cdk1 analysis, embryos were fixed and staged as described [S5]. For phosphatase treatment, ten staged embryos were transferred to 10 μl phosphatase buffer and either 0.01 U calf intestinal phosphatase (New England Biolabs) or phosphatase inhibitors (1 mM NaVO4, 10 mM NaF, and 80 mM β-glycerolphosphate), then incubated at 37°C for 30 min. For Western blots, ten embryos/lane were loaded, and antibodies were used at the following dilutions: rabbit anti-pY15cdk1, 1:1000 (Cell Signaling Technology) and rabbit anti-cdk1, 1:1000-1:2500 (gift of Pat O'Farrell).

Immunofluorescence

Embryos were dechorionated and fixed as described [S6]. Embryos were stained with the following primary antibodies: mouse monoclonal anti-α-tubulin, 1:100 (Sigma); rabbit polyclonal anti-γ-tubulin, 1:100 (Sigma); and rabbit polyclonal anti-Dgrip84, 1:100 [S7]. Secondary antibodies conjugated to FITC or rhodamine (Jackson Labs) were used at 1:500. DNA was stained with 10 μg/ml bisbenzimide (Molecular Probes) for 5 min. Embryos were analyzed on a Leica DMR microscope with a Sensicam CCD camera and Slidebook software (Intelligent Imaging Innovations). Division cycle number was determined by nuclear density as previously described [S5].

Time-Lapse Confocal Microscopy

Embryos were collected for 30 min, aged for 30 min, dechorionated in 50% bleach, and mounted on coverslips in halocarbon oil 700 (Sigma). Embryos were visualized with a Leica DMRXA microscope equipped with a spinning disk confocal laser system (Perkin Elmer) and a CCD camera (Hamamatsu). Division cycle number was determined by nuclear density as previously described [S5].

Brain Squashes

Brain squashes were carried out as described before [S8] with wandering third-instar larvae. DNA was stained with 10 mg/ml bisbenzimide (Molecular Probes) for 10 min. Statistical significance was determined by Student's t test.

Supplemental References

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