JCB
Online Supplemental Material

Hauf et al. http://www.jcb.org/cgi/doi/10.1083/jcb.200208092

Kinase assay

For the experiment in Fig. 2 D, HeLa cells were arrested for 16 h in nocodazole. For the Cdk1 kinase assay, cells were lysed (20 mM Tris, pH 7.5, 250 mM NaCl, 2 mM EGTA, 1 mM DTT, 1% Triton X-100, 20 mM β-glycerophosphate, 1 mM NaF, protease inhibitors [leupeptin, chymostatin, and pepstatin at a concentration of 10 μg/ml]). A low speed supernatant of this extract was precleared with empty GammaBind Plus Sepharose beads (Amersham Biosciences). The protein concentration was determined by Bradford assay and adjusted to 5 μg/μl with lysis buffer. Monoclonal mouse anti–cyclin B1 antibody (GNS1), or as a control, mouse anti-myc (9E10), was coupled to GammaBind Plus Sepharose in a ratio of 1 mg antibody per 5 ml beads. The antibody beads were rotated over-end in the HeLa extract for 90 min at 4°C and then washed three times with TBS-T supplemented with phosphatase inhibitors (40 mM NaF, 20 mM β-glycerophosphate). A ratio of 10 μl beads per 500 μg of extract was used for immunoprecipitation. Beads were aliquoted for the kinase assay and washed once with kinase buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM NaF). The kinase assay was performed with 10 μl beads in 20 μl kinase buffer containing 5 μg histone H1 (Roche), 1 μM ATP, 1 μCi [γ-^{32}P]ATP, and the appropriate concentration of Hesperadin or DMSO for 30 min at 37°C. SDS sample buffer was added, and samples were boiled and resolved by SDS-PAGE. The gel was dried, and the radioactive signal was detected by PhosphorImager (Amersham Biosciences) analysis. The data was analyzed using ImageQuant software (Amersham Biosciences).

For the Aurora B kinase assay, HeLa cells were lysed in buffer containing 50 mM NaCl. The whole cell extract was spun at 13,000 rpm for 20 min at 4°C using a table top centrifuge. The pellet obtained from 200 mg of whole cell extract was extracted again in 15 ml lysis buffer containing 250 mM NaCl in order to obtain active Aurora B kinase from mitotic chromatin. The low speed supernatant of the latter extract was used for immunoprecipitation. Monoclonal mouse anti–AIM-1, or mouse anti-HA, was coupled to GammaBind Plus Sepharose, and beads were rotated over-end in the extract for 90 min at 4°C. Beads were washed, aliquoted, and washed in kinase buffer as for the Cdk1 kinase assay. The kinase assay was performed with 10 μl beads in 20 μl kinase buffer containing 5 μg histone H3 (Roche), 10 μM ATP, 2.5 μCi [γ-^{32}P]ATP, and the appropriate concentration of Hesperadin or DMSO for 20 min at 37°C. Subsequent analysis was as for Cdk1.

Nocodazole arrest/release experiments

For the experiment in Fig. 5, A and C, HeLa cells were treated with 330 nM nocodazole for 30 min, and mitotic cells were harvested by shake off. The shake-off procedure took ~25 min, and all cells were therefore pooled before replating. Cells were cultured for an additional 30 min in nocodazole, and then 50 nM Hesperadin or a corresponding concentration of DMSO (0.01%) was added. Cells were cultured for another 30 min, one sample was taken, and the remaining cells were washed twice with full medium containing Hesperadin or DMSO, but no nocodazole, and then plated in such medium. The washing procedure took 45 min, and one sample was taken directly after washing (0'). Further samples were harvested after specified amounts of time, and all were analyzed by chromosome spreading.

For the experiment in Fig. 5, B and D, HeLa cells were treated with 50 nM Hesperadin or a corresponding concentration of DMSO (0.01%). After 1 h, nocodazole was added, and mitotic cells were harvested by shake off 30 min later. Cells were cultured in Hesperadin/nocodazole or DMSO/nocodazole for another hour. To remove Hesperadin/DMSO, cells were washed twice with medium containing nocodazole, but no Hesperadin/DMSO, and then replated in medium containing nocodazole. The washing procedure took 40 min. Subsequently, cells were cultured for another hour, one sample was taken, and nocodazole was washed out from the remaining samples by washing twice with medium. Washing took 30 min, and one sample was taken immediately after the washing procedure (0'). Further samples were harvested after specified amounts of time, and all were analyzed by chromosome spreading.

Detailed specifications for immunofluorescence staining

PtK1 or PtK2 cells (Fig. 7; Fig. S3) were grown on coverslips, extracted in 0.5% Triton X-100 in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM $MgSO_4$, pH 7.0) for 5 min, fixed in 4% paraformaldehyde in PHEM for 7 min, rinsed in 0.05% Tween 20 in PBS, and blocked in 5% FCS in PHEM for 45 min. Antibody incubations were in 5% FCS in PHEM, rinsings in PBS/0.5% Tween 20. Cells were mounted in 5 mg/ml p-phenylenediamine in 20 mM Tris, pH 8.8/80% glycerol.

For the experiment in Fig. 9, HeLa cells were spun onto glass slides using a cytospin centrifuge (Shandon). After centrifugation, cells were extracted in 0.25% Triton X-100/0.1% BSA/10 μM nocodazole in PBS (for Hesperadin-treated samples, Hesperadin was also added at 100 nM) for 2.5 min at room temperature. Cells were then fixed in 4% paraformaldehyde in PBS for 7 min, incubated for 5 min in 0.1% BSA in PBS, and subsequently incubated with antibodies in 0.1% BSA/PBS.

Spindle assembly checkpoint overcome experiments

For the experiment in Fig. 8, A–D, HeLa cells were treated with 330 nM nocodazole or 10 μM taxol for 1 h. Mitotic cells were harvested by shake off, replated, and cultured for an additional 1 h in the presence of nocodazole or taxol. One sample was harvested. 100 nM Hesperadin or a corresponding concentration of DMSO (0.02%) was added to the culture medium. Samples were harvested after certain amounts of time and analyzed by chromosome spreading. Cells treated with monastrol (100 μM) were processed in the same way, except that incubation before mitotic shake off was for 2 h and cells were replated onto poly-L-lysine coated coverslips, fixed with paraformaldehyde at appropriate time points, and analyzed by immunostaining.

Live cell imaging of HeLa H2B–GFP cells

For the experiment shown in Video 1, HeLa H2B–GFP cells (Kanda, T., K.F. Sullivan, and G.M. Wahl. 1998. *Curr. Biol.* 8: 377–385) were grown on coverslips and treated with 100 nM Hesperadin for up to several hours before filming. Cells were observed with a Carl Zeiss MicroImaging, Inc. Axiovert 100M microscope equipped with an incubation chamber (Carl Zeiss MicroImaging, Inc.) to keep cells at 37°C and 5% CO_2. Images were recorded with a CoolSnap FX camera (Photometrics) using a 40× or 63× objective and processed using MetaMorph software. For the cell shown in the first part of the movie, one Z section was recorded in 2–5-min interval. For the cell shown in the second part, four Z sections spaced by 5 μm were recorded every 3 min and overlaid to produce the image shown in the upper part.