Cdk1 and Okadaic Acid-sensitive Phosphatases Control Assembly of Nuclear Pore Complexes in *Drosophila* Embryos^V

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Disassembly and reassembly of the nuclear pore complexes (NPCs) is one of the major events during open mitosis in higher eukaryotes. However, how this process is controlled by the mitotic machinery is not clear. To investigate this we developed a novel *in vivo* model system based on syncytial *Drosophila* embryos. We microinjected different mitotic effectors into the embryonic cytoplasm and monitored the dynamics of disassembly/reassembly of NPCs in live embryos using fluorescently labeled wheat germ agglutinin (WGA) or in fixed embryos using electron microscopy and immunostaining techniques. We found that in live embryos Cdk1 activity was necessary and sufficient to induce disassembly of NPCs as well as their cytoplasmic mimics: annulate lamellae pore complexes (ALPCs). Cdk1 activity was also required for keeping NPCs and ALPCs disassembled during mitosis. In agreement recombinant Cdk1/cyclin B was able to induce phosphorylation and dissociation of nucleoporins from the NPCs *in vitro*. Conversely, reassembly of NPCs and ALPCs was dependent on the activity of protein phosphatases, sensitive to okadaic acid (OA). Our findings suggest a model where mitotic disassembly/reassembly of the NPCs is regulated by a dynamic equilibrium of Cdk1 and OA-sensitive phosphatase activities and provide evidence that mitotic phosphorylation mediates disassembly of the NPC.

INTRODUCTION

The double lipid membrane of the nuclear envelope (NE) forms a border between the nucleus and the cytoplasm. The nuclear pore complexes (NPCs) are multiprotein channels located at the fusion points between the outer and inner nuclear membrane. Directional signal-mediated transport of macromolecules and free diffusion of small molecules between the nucleus and the cytoplasm occur through the NPCs. The framework of the NPC has an evolutionary conserved octagonal channel structure spanning the NE and consists of multiple copies of ~30 different proteins called nucleoporins (Rout *et al.*, 2000; Cronshaw *et al.*, 2002), most of which are conserved in higher eukaryotes (Mans *et al.*, 2004). About a third of the nucleoporins contain phenylalanine-glycine (FG) repetitive domains, which are believed to be involved in the selective permeability of the NPC (Weis, 2003; Peters, 2005). In mammals the majority of the FG-nucleoporins are modified with O-linked *N*-acetyl- α -glucosamine residues (Snow *et al.*, 1987), whose function is still

unknown. In addition to NPCs some cell types, such as oocytes and early embryonic cells, also contain mimics of NPCs inserted in cytoplasmic annulate lamellae (AL) membranes (Kessel, 1992). The annulate lamellae pore complexes (ALPCs) have a similar structure and nucleoporin composition as the NPCs (Meier *et al.*, 1995; Cordes *et al.*, 1996; Imreh and Hallberg, 2000; Miller and Forbes, 2000).

During the open mitosis of higher eukaryotes the NPCs (as well as the ALPCs) disassemble into distinct soluble nucleoporin subcomplexes (Macaulay *et al.*, 1995; Cordes *et al.*, 1996; Belgareh *et al.*, 2001), whereas the pore membrane proteins diffuse throughout the ER network (Yang *et al.*, 1997; Imreh and Hallberg, 2000; Daigle *et al.*, 2001). Disassembly of both types of pore complexes is accompanied by phosphorylation of a subset of the nucleoporins (Macaulay *et al.*, 1995; Favreau *et al.*, 1996; Ganeshan and Parnai, 2000; Belgareh *et al.*, 2001). At mitotic exit the NPCs sequentially reassemble in the reforming NEs of the daughter nuclei (Bodoor *et al.*, 1999; Belgareh *et al.*, 2001; Daigle *et al.*, 2001).

Mitotic events are controlled by a family of evolutionary conserved cyclin-dependent kinases (Cdks), which are enzymatically active only in a complex with regulatory cyclin subunits (reviewed in Doree and Hunt, 2002). Cdk1, which forms an active kinase complex with B-type cyclins plays an essential and specific role in triggering the mitotic events (Riabowol *et al.*, 1989; Beckhelling *et al.*, 2003). The activity of Cdks is regulated by the synthesis and destruction of the cyclins as well as by activating and inhibitory phosphorylation (reviewed in Obaya and Sedivy, 2002). Interestingly, some of the nucleoporins phosphorylated during mitosis were shown to be substrates for Cdk1 (Macaulay *et al.*, 1995;

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Abbreviations used: AL, annulate lamellae; ALPC, annulate lamellae pore complex; NE, nuclear envelope; NPC, nuclear pore complex; OA, okadaic acid; WGA, wheat germ agglutinin.

Favreau *et al.*, 1996; Ganeshan and Parnaik, 2000). However, at the moment very little is known about how the mitotic machinery controls disassembly and reassembly of the NPCs.

After fertilization the *Drosophila* embryo represents a multinucleated cell (syncytium) where embryonic nuclei rapidly (every 10 min) and synchronously divide for 13 rounds without undergoing cytokinesis (Foe and Alberts, 1983). Syncytial embryos contain an excessive pool of maternally contributed nucleoporins, which assemble into NPCs and ALPCs during interphases and disassemble during mitosis (Stafstrom and Staehelin, 1984; Kiseleva *et al.*, 2001; Onischenko *et al.*, 2004). From the 9th division cycle the nuclei are aligned 10–20 μ m beneath the embryonal periphery where mitotic divisions can be easily followed directly in living embryos (Foe and Alberts, 1983). Here we used early *Drosophila* embryos as a model system to dissect the mechanism regulating mitotic disassembly/reassembly of the NPCs.

MATERIALS AND METHODS

Fly Stocks and Collection of Embryos

The *Canton-S* strain was used as a wild-type in all the experiments. For heat-inducible inactivation of Cdk1 we used the *Cdk1^{ts}* (*A171T*) strain (Sigrist *et al.*, 1995) obtained from Dr. Christian Lehner. The mutant fly stock *png¹⁷²* (Shamanski and Orr-Weaver, 1991; Fenger *et al.*, 2000) was obtained from Dr. Terry Orr-Weaver. The embryos were collected at 22°C on agar plates, studied with dry yeast, and harvested according to (Allis *et al.*, 1977). The

embryos were batch-dechorionated with Clorox according to (Elgin and Hood, 1973). For time-lapse microscopy of live embryos they were manually dechorionated on a scotch tape.

Immunostaining

Except otherwise mentioned all the procedures were performed at RT. Untreated png embryos (see Figure 3) were fixed in the equal volumes of *n*-heptane and -70°C methanol. Microinjected png embryos (see Figure 6) were fixed for 20 min in 1:1 mixture of *n*-heptane and 3.7% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4, and subsequently freed from vitelline membranes with thin needles. Fixed embryos were permeabilized for 1 h with a blocking solution (0.5% bovine serum albumin [BSA] in PBS containing 0.1% Tween-20) + 0.1% Triton X-100 and subsequently incubated overnight at $+4^{\circ}$ in the blocking solution containing RL1 antibodies (Affinity Bioreagents, Golden, CO) diluted 1:5000 or anti-cyclin B antibodies F2F4 (Hybridoma Bank, Iowa City, IA) diluted 1:100, used as primary antibodies. The embryos were subsequently washed and incubated with 0.1 $\mu\text{g}/\text{ml}$ WGA-Alexa594 (Molecular Probes, Eugene, OR) and 1:2000 dilution of FITC donkey anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) or FITC goat anti-mouse antibodies (Molecular Probes), used as secondary antibodies. For visualization of nuclei the embryos were treated for 1 h with 1 mg/ml RNase-A in PBS-T (0.1% Tween-20 in PBS), followed by staining with 15 $\mu\text{g}/\text{ml}$ propidium iodide. The embryos were mounted in Vectashield media (Vector Laboratories, Burlingame, CA) and imaged as described in *Microinjections and Time-lapse Microscopy* but using Leica PL APO 635/1.32 oil objective (Deerfield, IL).

Western Blotting

The proteins, separated by SDS-PAGE, were transferred to nitrocellulose membranes. The membranes were incubated with anti-cyclin B antibodies diluted 1:300, RL1 antibodies diluted 1:1000, or a mixture of anti-gp210 antibodies AGP-26 and AGP-78 (Hybridoma bank) diluted 1:20 in PBS-T containing 5% dry milk. After washes the membranes were probed with HRP-conjugated goat anti-rabbit or sheep anti-mouse antibodies (Amersham-

Figure 1. Time-lapse microscopy of the distribution of microinjected WGA during syncytial nuclear divisions in *Drosophila* embryos. (A and Supplementary Video 1) Time-lapse confocal microscopy of a 10th division cycle *Drosophila* embryo after injection of a 0.1 mg/ml solution of WGA-Alexa594. The distribution of WGA-Alexa was monitored for 20 min (the duration of 2 division cycles). Note the periodical oscillations between intense staining of cytoplasmic foci and nuclear peripheries and diffuse localization. (B) Dual wavelength time-lapse microscopy of a 12th cycle embryo microinjected with a combination of (0.1 mg/ml) WGA-Alexa594 (top row) and (4 mg/ml) NLS-GFP (bottom row). Note that during mitosis NLS-GFP dispersed throughout the cytoplasm, but rapidly reaccumulated in the newly formed interphase daughter nuclei. Scale bars, 10 μm .

Figure 2. Effects of cycloheximide and cyclin B on disassembly of NPCs and ALPCs in wild-type syncytial embryos. (A) Western blot analysis of cyclin B in embryonal lysates 20 min after microinjection of cycloheximide or buffer. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies specific for *Drosophila* cyclin B. Ponceau-S staining was used as a loading control. (B and Supplementary Video 2) Time-lapse confocal microscopy of syncytial embryos injected with cycloheximide. WGA-Alexa594-labeled nuclear rims and cytoplasmic foci persisted for more than 20 min, indicating that ALPCs and NPCs failed to disassemble. Note that the embryonic nuclei did not divide, but moved out of the focal plane. This is better appreciated in the corresponding video (Supplementary Video 2). (C–G) EM analysis of cycloheximide-injected embryos. All cycloheximide injected embryos ($n = 6$) contained typical interphase nuclei (C) with intact NPCs (D) and abundant cytoplasmic AL (E) with normal ALPCs (F). (G) EM morphometric quantification of ALPCs. The vertical bars show the total number of ALPCs in six randomly selected cycloheximide-injected embryos and in four buffer-injected interphase embryos (control), respectively. Note that the embryos injected with cycloheximide contain more ALPCs (mean value, 7.72×10^6 ALPCs/embryo) than the buffer-injected interphase embryos (mean value, 4.07×10^6 ALPCs/embryo). (H and Supplementary Video 3) Time-lapse confocal microscopy of syncytial embryos injected with cycloheximide together with GST-cyclin B. WGA-Alexa594 displayed a permanent uniform distribution characteristic for mitotic embryos with disassembled pore complexes. (I–K) EM analysis of embryos coinjected with cycloheximide and GST-cyclin B. After 20-min incubation all cycloheximide + GST-cyclin B injected

Pharmacia Biotech, Piscataway, NJ) used as secondary antibodies. For probing with WGA we blocked the membranes with 0.1% gelatin from porcine skin (Sigma, St. Louis, MO) in PBS-T and incubated them with 0.4 $\mu\text{g}/\text{ml}$ HRP-conjugated WGA (Vector Laboratories). Western blots were developed in ECL detection reagent (Amersham Pharmacia Biotech) and analyzed using the Luminescent Image Analyzer, Las1000plus system (Fuji Photo Film Co., Tokyo, Japan). The images were processed by Adobe PhotoShop 7.0 software (Adobe Systems, San Jose, CA).

Microinjections and Time-lapse Microscopy

The embryos collected for 2 h were injected with a volume, corresponding to ~1–5% of the total embryonal volume, using inverted microscope with air-driven Microinjector 5242 (Eppendorf, Fremont, CA) under halocarbon oil. For experiments performed at 22°C embryos were covered with 1:1 mixture of Halocarbon oil 700 and Halocarbon oil 27 (Sigma), whereas for the experiments performed at 30°C they were covered with pure Halocarbon oil 27 to increase the oxygen supply. For studies of living embryos the slides with the injected embryos were placed directly under the microscope equipped with *The Cube* conditioning system (Life Imaging Services, Reinach, Switzerland). Images were acquired with Leica TCS-SP confocal microscope equipped with Leica Cofocal Software 2.61 and Leica PL APO 405/1.25 oil objective and processed with Adobe PhotoShop 7.0 software (Adobe Systems). For immunostaining and EM studies the injected embryos were incubated in a humid chamber at the appropriate temperature, released from the slides with *n*-heptane, and transferred either into glutaraldehyde-based fixative (for EM), or into formaldehyde fixative (for immunostaining).

Cycloheximide and colchicine (Sigma-Aldrich) were added at final concentration of 1 mg/ml to injection buffer (0.5× PBS, pH 7.4). Roscovitine (Calbiochem, San Diego, CA) and okadaic acid (Sigma-Aldrich) were injected at final concentrations of 5 mM and 150 μM , respectively. In the case of roscovitine we also added dimethyl sulfoxide to the injection solutions up to 20% to increase the solubility. *Drosophila* GST-cyclin B purified according to Carroll *et al.* (1998) from the bacterial expression construct (a gift of Dr. Douglas Kellogg) was added to the microinjection solutions at concentrations up to 7.5 mg/ml. For visualization of the NPCs and ALPCs in live embryos we microinjected WGA-Alexa594 (0.1 mg/ml), resulting in a final concentration of WGA inside the embryo of ~0001–0005 mg/ml, which is far below the concentrations reported to affect nuclear import and pore complex assembly (Finlay *et al.*, 1987; Dabauvalle *et al.*, 1990).

Electron Microscopy and EM-Morphometry

The embryos, fixed and stained en bloc with uranyl acetate essentially according to Zalokar and Erk (1977), were dehydrated with ethanol and embedded individually into Agar-100 according to the standard protocol. The ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with a JEM-100 electron microscope (JEOL, Peabody, MA).

For morphometric analysis, individual embryos were sectioned in the equatorial plane. To determine the total number of ALPCs per embryo, we counted the numbers of ALPCs directly under the electron microscope in 10 randomly selected areas (504 μm^2) of the embryo section. These data and the diameter of the ALPC (0.12 μm) were used to calculate the average volume density (VD) of ALPCs in each embryo by standard stereologic equations (Gruzdev, 1974). The volume (V) of the embryo was determined through its horizontal and vertical dimensions (measured under the light microscope in the semithin sections), assuming an elliptical shape. The total number of ALPCs in the embryos was calculated by multiplying V with VD.

In Vitro Assay for NPC Disassembly

The embryonic nuclei were purified from fresh 3–7-h old embryos essentially as described in Fisher *et al.* (1982), except that for homogenization of embryos and washes of nuclei we used 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 50 mM

NaCl, 250 mM sucrose, 1 mM dithiothreitol (DTT). The nuclei were washed an additional two times with the same buffer, resuspended in 1 volume (initial volume of embryos) of 20 mM Tris-HCl, pH 7.4, and 5 mM MgCl₂ and kept on ice. Two microliters of the freshly prepared nuclear suspension was mixed with 30 μl of the reaction buffer (50 mM KCl, 40 mM β -glycerophosphate, 10 mM EGTA, 5 mM MgCl₂, 2 mM DTT, 0.2% BSA, 20 mM Pipes, pH 7.4, with the addition of 1/20 volume of ATP-regenerating system). The disassembly reaction was performed for 1 h at RT in the presence of 1 μg (10 μl) of active recombinant human Cdc2-cyclin B complex (Upstate Biotechnology, Lake Placid, NY). The reactions were centrifuged at 100,000 × *g* in a fixed-angle rotor for 10 min at 4°C. The supernatants were mixed 1:1 with sample buffer. The pellets were resuspended and diluted in the reaction buffer to the equal volumes as supernatants and mixed 1:1 with sample buffer.

RESULTS

Microinjected Fluorescently Labeled WGA Can Be Used as an In Vivo Marker for NPCs and ALPCs in *Drosophila* Embryos

Previously we have shown (Onischenko *et al.*, 2004) that wheat germ agglutinin (WGA) specifically bound to only one *Drosophila* NPC protein, NUP58, and perfectly colocalized with two well-established monoclonal nucleoporin marker antibodies (mAb414; Davis and Blobel, 1986 and RL1; Snow *et al.*, 1987). In addition, microinjected gold-labeled WGA specifically decorated the central portion of the NPCs and ALPCs in syncytial embryos analyzed by EM. We wanted to exploit the use of WGA as marker for NPCs and ALPCs in live *Drosophila* embryos. For this, we microinjected 0.1 mg/ml fluorescently labeled WGA (WGA-Alexa594) into live early embryos and followed its distribution with time by confocal laser scanning microscopy. During interphase syncytial embryos displayed brightly labeled NEs and cytoplasmic foci (Figure 1A and Supplementary Video 1) in agreement with the distribution of WGA to the NPCs and the ALPCs. The nuclear rim staining and the dotted pattern in the cytoplasm synchronously disappeared during mitosis and reappeared in telophase, consistent with the timing of disassembly and reassembly of NPCs and ALPCs (Stafstrom and Staehelin, 1984; Onischenko *et al.*, 2004). Usually the staining of the NEs was more intense than the staining of the cytoplasmic foci either because of lower levels of NUP58 or lower accessibility for WGA in the AL membrane stacks. Microinjection buffers containing 0.1 mg/ml WGA-Alexa can be safely used for getting a quick qualitative assessment of disassembly/reassembly of NPC and ALPC in live *Drosophila* embryos, since we did not observe any abnormalities in nuclear import (Figure 1B), timing of mitotic divisions, or embryonic development (unpublished data). At 0.2 mg/ml some effects on nuclear import and nuclear growth were observed locally at the area of injection.

Mitotic Cyclins Are Required for Disassembly of NPCs and ALPCs in Syncytial *Drosophila* Embryos

In a previous study we reported that the major fraction of excess nucleoporins in syncytial *Drosophila* embryos remains soluble and that one of the nucleoporins (p150) is hyperphosphorylated (Onischenko *et al.*, 2004). Because syncytial embryos are known to display high levels of mitotic cyclins and Cdk1 activity (Edgar *et al.*, 1994), we were interested in investigating how mitotic factors influence disassembly/reassembly of pore complexes. We microinjected syncytial embryos with the protein synthesis inhibitor cycloheximide, reported to cause depletion of mitotic cyclins (Edgar *et al.*, 1994). As expected, cycloheximide quickly reduced the level of cyclin B (Figure 2A). Cycloheximide also completely blocked disassembly of NPCs and ALPCs in the injected embryos as shown using time-lapse microscopy (Figure 2B

Figure 2 (cont). embryos (*n* = 5) displayed concentric fenestrated membranes around the chromatin (I), apparently lacking NPCs (J). (K) EM-morphometry of five randomly selected embryos microinjected with cycloheximide + GST-cyclin B and of five buffer injected mitotic embryos (controls). Note that cycloheximide + GST-cyclin B-injected embryos contain similar amounts or even less ALPCs (mean value, 0.39×10^6 ALPCs/embryo) than the control group (mean value, 0.84×10^6 ALPCs/embryo). (L) Western blot analysis of phosphorylation of p150 in the embryos injected with cycloheximide, buffer, or cycloheximide + GST-cyclin B. Equal amounts of lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with RL1 to visualize nucleoporin p150. Note the increased presence of the faster migrating dephosphorylated form of p150 (arrow) in cycloheximide-injected embryos, which was absent in embryos injected with cycloheximide + GST-cyclin B. Scale bars, (B and H) 10 μm ; (C, E, and I) 1 μm ; (D, F, and J) 0.5 μm .

Figure 3. Characterization of NPCs and ALPCs in *png* mutant embryos. (A) Confocal images of a 0–4-h old homozygous *png* mutant embryo fixed and immunostained with WGA and monoclonal RL1 antibodies. In all embryos ($n > 100$) WGA and RL1 specifically colocalized at the NEs of a few giant nuclei (labeled with “n”) and at multiple large cytoplasmic foci. (B) Fixed embryo double-stained with WGA (green) and propidium iodide (red) to visualize chromatin of giant nuclei. (C–F) EM analysis of *png* embryos. A portion of the NE of a giant nucleus (C) with apparently intact NPCs (D). The cytoplasm of the embryos was filled with giant stacks of AL (E) containing densely packed ALPCs (F). Scale bars, (A) 20 μm ; (B and D) 5 μm ; (C and E) 0.5 μm .

and Supplementary Video 2). This conclusion was supported by EM analysis because all cycloheximide-injected embryos ($n = 6$) contained typical interphase nuclei with intact NPCs (Figure 2, C and D) and abundant ALPCs (Figure 2, E and F). Quantification of ALPCs using EM-morphometry showed that cycloheximide-injected embryos actually contained more ALPCs (about twofold) than interphase control embryos, injected with buffer (Figure 2G). The effect of cycloheximide was primarily due to depletion of mitotic cyclins, since it could be completely reversed by coinjection with nondegradable recombinant *Drosophila* GST-cyclin B (Su *et al.*, 1998) resulting in persistently disassembled NPCs and ALPCs as judged by time-lapse microscopy of live embryos (Figure 2H and Supplementary Video 3), by EM analysis (Figure 2, I and J) and morphometric quantification of ALPCs (Figure 2K). Furthermore, cycloheximide injection also caused massive dephosphorylation of the nucleoporin p150, which is normally dephosphorylated when assembled into NPCs and ALPCs during interphase (Onischenko *et al.*, 2004). The cycloheximide-dependent dephosphorylation of p150 was completely abolished by coinjection of GST-cyclin B (Figure 2L). The results indicate that in normal syncytial embryos disassembly of NPCs and ALPCs is dependent on mitotic cyclins.

If disassembly of pore complexes requires mitotic cyclins one would expect impaired disassembly of pore complexes in syncytial embryos obtained from *png* mutant flies, which have low endogenous levels of the major mitotic cyclins A and B (Fenger *et al.*, 2000). Indeed, in all *png* embryos immunostaining with WGA and RL1 antibodies revealed intense labeling of the NEs of a few giant nuclei unable to divide (Lee *et al.*, 2001), as well as abundant cytoplasmic structures resembling AL (Figure 3, A and B). This was confirmed by EM analysis, which revealed assembled NPCs (Figure 3, C and D) as well as unusually large stacks of AL with tightly packed ALPCs (Figure 3, E and F). The number

of ALPCs in *png* mutants was even higher than in the cycloheximide-injected embryos (for EM-morphometry; see Figure 4C). The large quantity of persistently assembled ALPCs in cyclin-deficient *png* mutants supports the idea that disassembly of NPCs and ALPCs requires mitotic cyclins.

Cdk1 Activity Mediates Disassembly of NPCs and ALPCs in Syncytial Drosophila Embryos

To further dissect the mechanism behind pore complex disassembly in *Drosophila* embryos we used the cyclin-deficient *png* mutants. As expected, time-lapse movies of buffer-injected *png* embryos displayed abundant persistently assembled ALPCs in the syncytial cytoplasm (Figure 4A and Supplementary Video 4), which quickly disassembled after microinjection with GST-cyclin B (Figure 4B and Supplementary Video 5). Morphometric analysis of embryos which were fixed and prepared for EM (Figure 4C) showed that the number of ALPCs were ~ 15 -fold lower in *png* embryos injected with GST-cyclin B (mean value, 0.73×10^6 ALPCs/embryo) than that of buffer-injected *png* control embryos (mean value, 12.1×10^6 ALPCs/embryo). The cyclin B induced disassembly of ALPCs was completely blocked by coinjection with the specific Cdk inhibitor roscovitine (Figure 4D), indicating that in cyclin-deficient embryos exogenous cyclin B is able to promote disassembly of pore complexes by activating Cdk1 (Campbell *et al.*, 1995; Jacobs *et al.*, 1998).

This led us to investigate the role of Cdk1 in regulation of pore complex disassembly/reassembly. We took advantage of embryos, bearing a point mutation in Cdk1, which makes the enzyme thermosensitive (Sigrist *et al.*, 1995). The activity of Cdk1 can be efficiently blocked in the Cdk1^{ts} embryos by raising the temperature from 22 to 30°C. Time-lapse movies of WGA-Alexa injected Cdk1^{ts} embryos showed that at 30°C NPCs and ALPCs did not disassemble (Figure 4E, top row,

Figure 4. Effects of cyclin B and Cdk1 on disassembly of NPCs and ALPCs. (A, B, and D) Time-lapse microscopy of ALPC assembly in png mutant embryos visualized with WGA. Embryos, 0–4 h old, derived from homozygous png mutant females were microinjected with buffer (A), GST-cyclin B (B), or GST-cyclin B + roscovitine (D). ALPCs failed to disassemble in buffer-injected control embryos (A and Supplementary Video 4), but totally dispersed 10 min after injection with GST-cyclin B (B and Supplementary Video 5). (C) EM morphometric analysis of the number of ALPCs in embryos injected with buffer or GST-cyclin B, respectively. Disassembly was completely suppressed in embryos coinjected with GST-cyclin B and the Cdk1 inhibitor roscovitine (D). (E) Assembly of NPCs and ALPCs in live Cdk1^{ts} mutant embryos was monitored by time-lapse confocal microscopy of WGA distribution. At 30°C, when Cdk1 is inactivated, disassembly of NPCs and ALPCs is suppressed (E, top row, and Supplementary Video 6). By contrast, in Cdk1^{ts} embryos at 22°C (E, middle row, and Supplementary Video 7), as well as in wild-type embryos incubated at 30°C (E, bottom row), NPCs and ALPCs normally disassembled. (F–H) EM analysis showing a typical nucleus (F) of a CDK1^{ts} embryo incubated for 20 min at 30°C and an enlarged view a portion of the NE (G), with intact NPCs (arrows). (H) A representative image of cytoplasmic AL with intact ALPCs (arrows). (I) EM morphometric analysis of five randomly selected embryos from each group, showing that embryos with inactivated Cdk1 contained substantially more ALPC than any of the control embryos, indicating that ALPC disassembly is suppressed. Scale bars, (F) 1 μm ; (G and H) 0.5 μm .

Figure 5. The role of Cdk1 in disassembly of NPCs and ALPCs during mitosis. Time-lapse of Cdk1^{ts} embryos, injected with colchicine (A and Supplementary Video 8) or GST-cyclin B (B) and incubated at 22°C to arrest the nuclear divisions in mitosis. After 15 min (time 0:00) the temperature was shifted to 30°C for 20 min to monitor pore complex assembly. Note that NPCs and ALPCs reassembled after 10 min in Cdk1^{ts} embryos, but not in wild type embryos. Scale bar, 10 μ m.

and Supplementary Video 6). The inability to disassemble was specifically due to inactivation of Cdk1 because the pore complexes disassembled and reassembled normally in Cdk1^{ts} embryos incubated at 22°C (Figure 4E, middle row and Supplementary Video 7) and wild-type embryos incubated at 30°C (Figure 4E, bottom row). At the EM level all of the Cdk1^{ts} embryos at 30°C ($n = 6$) had intact NEs with assembled NPCs (Figure 4, F and G) and ALPCs (Figure 4H). The quantity of ALPCs in all Cdk1^{ts} embryos at 30°C ($n = 5$) was substantially higher than in any of the wild-type embryos at 30°C ($n = 5$) or Cdk1^{ts} mutants at 22°C ($n = 5$; Figure 4I). On the basis of our findings we conclude that Cdk1 activity is critically required for disassembly of pore complexes in *Drosophila* embryos.

To find out if the Cdk1 activity is only required to initiate disassembly, or if it is also necessary for keeping pore complexes in a disassembled state during mitosis, we first blocked embryonic division cycles in Cdk1^{ts} mutants or wild-type embryos (control) in mitosis by microinjection of either colchicine (Foe *et al.*, 2000) or GST-cyclin B (Su *et al.*, 1998). After 15 min of incubation, when nuclear divisions were arrested in mitosis and both NPCs and ALPCs were disassembled, we raised the temperature to 30°C and monitored pore complex reassembly by time-lapse microscopy of live embryos. In both cases we observed massive reassembly of the NPCs and ALPCs in Cdk1^{ts} embryos 5–10 min after shifting to the restrictive temperature, whereas wild-type embryos remained unaffected (Figure 5 and Supplementary Video 8). The results show that Cdk1 activity is continuously required in order to keep NPCs and ALPCs disassembled during mitosis.

Purified Recombinant Cdk1-Cyclin B Complex Is Able to Dissociate Nucleoporins from Purified Embryonic Nuclei In Vitro

To further dissect the mechanism behind Cdk1-dependent disassembly of pore complexes, we injected one pole of the cyclin-deficient png mutant embryos with GST-cyclin B followed by immediate fixation and immunostaining to study

the relation between distribution of cyclin B, ALPCs, and NPCs by immunofluorescence microscopy. The propagation of the cyclin B diffusion zone strictly correlated with the sharp border between diffuse WGA staining and fluorescing

Figure 6. Spatiotemporal correlation between cyclin B distribution and disassembly of NPCs and ALPCs. (A–C) Distribution of microinjected GST-cyclin B and assembled pore complexes in png mutant embryos. (A) In fixed microinjected embryos double-stained with WGA and anti-cyclin B antibodies the distribution of cyclin B (red) perfectly correlated with disassembly of ALPCs (green). (B and C) In embryos double stained with WGA (green) and propidium iodide (red) (used to visualize nuclei) ALPCs and NPCs present before (B) and absent after (C) the passage of the sharp GST-cyclin B diffusion zone. Scale bars, 50 μ m.

Figure 7. Recombinant Cdk1-Cyclin B can phosphorylate p150 and dissociate nucleoporins from the purified *Drosophila* nuclei in vitro. Purified embryonic nuclei were incubated at room temperature for 0 or 60 min in the absence or presence of human recombinant Cdk1-cyclin B and the specific Cdk1 inhibitor roscovitine, as indicated. After centrifugation at $100,000 \times g$, equal amounts of the supernatant (S) and pellet (P) fractions were separated by SDS-PAGE and analyzed by Western blotting. WGA and monoclonal RL1 antibodies were used to detect nucleoporins, whereas antibodies specific for gp210 were used to control for pelleting of the fraction still associated with the NE membranes. Note that Cdk1 activity was able to induce phosphorylation of p150 (arrows) as well as dissociation of p150 and four other nucleoporins from the nuclear membrane fraction.

nuclear rims and cytoplasmic foci (Figure 6), demonstrating that local cyclin B-induced Cdk1 activity is able to promote efficient disassembly of both NPCs and ALPCs.

This led us to examine if Cdk1 activity is able to directly dissociate nucleoporins from pore complexes. For this, we thoroughly purified embryonic nuclei from cytoplasmic components and incubated them with pure recombinant

Cdk1-cyclin B in the presence of an ATP-regenerating system. Cdk1-cyclin B induced solubilization of dNUP58 and all five RL1 interacting *Drosophila* nucleoporins including p150 (Figure 7). Cdk1-cyclin B also caused hyperphosphorylation of p150 (Figure 7). Both effects were completely blocked by the potent Cdk inhibitor roscovitine. The results show that phosphorylation of p150 and dissociation of the nucleoporins are both directly caused by Cdk1-cyclin B activity on the purified nuclei and points to an important role of phosphorylation in pore complex disassembly during mitosis.

Assembly of NPCs and ALPCs Depends on OA-sensitive Protein Phosphatase(s)

The requirement for persistently active Cdk1 for keeping pore complexes disassembled during mitosis, imply the existence of a reverse acting protein phosphatase(s), which continuously drive reassembly of NPCs and ALPCs. Because two major protein phosphatases, PP1 and PP2A, have been implicated in dephosphorylation of many Cdk1 substrates, we decided to investigate their possible role in driving NPC and ALPC reassembly. For this purpose we micro-injected embryos with okadaic acid (OA), which specifically and potently blocks phosphatases PP2A and PP1 (Takai *et al.*, 1987; Bialojan and Takai, 1988). Reassembly of NPCs and ALPCs was prevented in OA-injected Cdk1^{ts} mutant embryos incubated at 22°C (Figure 8A and Supplementary Video 9) as well as in OA-injected wild type embryos (unpublished data). To exclude the possibility that OA prevents reassembly of the pore complexes by affecting Cdk1 activity, we pretreated Cdk1^{ts} embryos with OA for 15 min at 22°C to allow complete disassembly and then inactivated Cdk1 at 30°C. This did not induce reassembly of ALPCs and NPCs (Figure 8B and Supplementary Video 10), demonstrating that reassembly of NPCs and ALPCs requires OA-sensitive protein phosphatase activity.

If the OA-sensitive protein phosphatases continuously drive reassembly of pore complexes during mitosis, one

Figure 8. Effect of okadaic acid on the assembly of NPCs and ALPCs. (A–C) Time-lapse microscopy of WGA-Alexa distribution in thermosensitive Cdk1^{ts} embryos microinjected with OA. (A and Supplementary Video 9) In embryos incubated at 22°C NPCs and ALPCs disassembled and completely disappeared after 10 min. (B and Supplementary Video 10) In embryos first incubated at 22°C for 15 min (to allow complete disassembly of NPCs and ALPCs) before shifting to 30°C (at time 0:00) NPCs and ALPCs failed to reassemble. (C) Time-lapse microscopy of embryos derived from png mutant flies microinjected with either OA alone or with OA + roscovitine. Note that OA-dependent disassembly of ALPCs was completely prevented by roscovitine. Scale bars, 20 μ m.

Figure 9. A schematic model of the regulation of disassembly and reassembly of pore complexes in *Drosophila* syncytial embryos. During the nuclear divisions disassembly and reassembly of pore complexes is regulated by a dynamic equilibrium of antagonistically acting Cdk1 and OA-sensitive phosphatases. One attractive possibility is that this process is directly regulated by phosphorylation and dephosphorylation of nucleoporins.

would expect OA treatment to induce disassembly of pore complexes when limited Cdk1 activity is normally insufficient to drive this process. This was indeed the case in png mutant embryos (which are deficient in Cdk1 activity compared with normal embryos) where microinjection of OA immediately induced massive disassembly of ALPCs (Figure 8C, top row). As expected this effect was suppressed by roscovitine (Figure 8C, bottom row). Taken together our findings suggest a model where the activities of Cdk1 and OA-sensitive phosphatase(s) antagonistically regulate disassembly and reassembly of pore complexes during mitosis.

DISCUSSION

Drosophila Embryos as a New Model System for Studies of Disassembly and Reassembly of the NPC

Most of the available data concerning postmitotic assembly of the NPC have been produced using a powerful in vitro model system based on *Xenopus* egg extracts (Lohka and Masui, 1983). However, complete understanding of this process requires complementary in vivo experimental system(s). Such systems should preferentially be based on one of the available genetic model organisms and should be easy to monitor and manipulate. Developing early *Drosophila* embryos very well fit these requirements. From an experimental perspective syncytial embryos can be regarded as a sample in a test tube containing a pool of excessive maternally contributed nucleoporins, which rapidly and synchronously oscillate between a disassembled state and a state of assembly in NPC and ALPC structures, respectively. Even during interphase in normal syncytial embryos the nucleoporins are only incompletely assembled (Onischenko *et al.*, 2004), making it possible to affect the system in the direction of increased or decreased assembly by manipulation of factors controlling the assembly and disassembly processes. The extent of pore complex assembly can be directly monitored in live embryos injected with fluorochrome-labeled WGA using time-lapse microscopy or visualized by immunofluorescence microscopy, by EM or quantified using EM-morphometric analysis. Assembly of NPCs and ALPCs in this system was sensitive to classical inhibitors of NE formation and pore complex assembly, such as BAPTA, GTP γ S, and NEM (unpublished data). Thus, the syncytial *Drosophila* embryo model appears to be compatible with other higher

eukaryotic models and, as demonstrated in this article, allows design of experiments to “push” the process of NPC disassembly/reassembly in either direction by microinjection of inhibitors or recombinant proteins or by using mutant fly strains.

Cdk1 and OA-sensitive Protein Phosphatases Are Mediators of Mitotic Pore Complex Disassembly/Reassembly

Cdk1 is a key mediator of pore complex disassembly, because selective inactivation of Cdk1 abolished disassembly of NPCs and ALPCs (Figure 4E), whereas activation of Cdk1 induced disassembly of NPCs and ALPCs (Figure 4B). According to our in vitro experiments recombinant Cdk1-cyclin B was able to induce hyperphosphorylation of the *Drosophila* nucleoporin p150 and dissociation of p150 together with at least four other nucleoporins from the NPCs of the purified nuclei. It is tempting to speculate that Cdk1-mediated phosphorylation of nucleoporins disrupts the interactions holding the structure together. This view is supported by the requirement for dephosphorylation of nucleoporins for assembly of pore complexes in vitro (Walther *et al.*, 2003). Although our data do not prove that Cdk1 phosphorylates nucleoporins directly, this view is consistent with the recently reported localization of Cdk1-cyclin B complex at the ALPCs just before the NE breakdown in *Xenopus* oocytes (Beckhelling *et al.*, 2003). Furthermore, in the filamentous fungus *Aspergillus nidulans*, which undergo closed mitosis, phosphorylation of a subset of nucleoporins was also shown to correlate with dissociation from the NPC, whereas the NPC remained functional (De Souza *et al.*, 2004). In this case phosphorylation of nucleoporins is carried out by the downstream serine/threonine kinase NIMA, which is activated by Cdk1. Although overexpression of NIMA apparently can induce NPC disassembly in mammalian cells (Lu and Hunter, 1995), attempts to identify a NIMA homologue in higher eukaryotes have been unsuccessful. It is possible that NIMA has a specialized function in reorganization of pore complexes in organisms undergoing closed mitosis. It should be pointed out that our data do not exclude the possibility that other kinases along with Cdk1 may also be involved in pore complex disassembly.

The reversibility of the *Drosophila* embryo model also allowed us to investigate the role of protein phosphatases in pore complex reassembly. Microinjection of OA at a concentration that specifically inhibits two major protein phosphatases, PP1 and PP2A, induced rapid disassembly of NPCs and ALPCs, suggesting that either or both of these enzymes are involved in postmitotic reassembly of the pore complexes. The involvement of PP1 and/or PP2A in pore complex reassembly is consistent with a number of evidence of their role in promoting postmitotic changes in the cell. For instance, in cultured cells excess PP1 promoted mitotic exit, whereas microinjection of anti-PP1 antibodies led to mitotic arrest (Fernandez *et al.*, 1992). Additionally, PP1 was implicated in postmitotic reassembly of the nuclear lamina (Thompson *et al.*, 1997; Steen *et al.*, 2000), whereas PP2A was shown to play an important role in postmitotic reassembly of Golgi (Lowe *et al.*, 2000).

Taken together our studies define Cdk1 and OA-sensitive protein phosphatases as the key regulators of pore complex disassembly/reassembly. However this by no means excludes involvement of other factors. Two recent studies performed using the *Xenopus* in vitro assembly system identified Importin- β as a negative regulator of pore complex assembly (Harel *et al.*, 2003; Walther *et al.*, 2003). The latter study also identified Ran-GTP as a positive regulator of this

process possibly acting via release of Importin- β from the nucleoporin subcomplexes (Walther *et al.*, 2003). Consistent with this our preliminary studies indicated that RanT24N and the nucleoporin-binding domain of importin- β both inhibited assembly of NPCs and ALPCs in live syncytial embryos (unpublished results).

Disassembly/Reassembly of Pore Complexes Is Regulated by a Dynamic Equilibrium between the Activities of Cdk1 and OA-sensitive Protein Phosphatases

The oscillation of pore complexes between a disassembled and an assembled state during syncytial nuclear divisions enables determination of dynamic aspects of pore complex disassembly/reassembly. Using this advantage, we were able to demonstrate that: 1) inactivation of Cdk1 during mitosis results in immediate reassembly of NPCs and ALPCs (Figure 5); 2) reassembly of NPCs and ALPCs is blocked by OA, a specific PP1/PP2A inhibitor (Figure 7, A and B); 3) in png mutant embryos, displaying persistently assembled NPCs and ALPCs (due to low endogenous Cdk1 activity, which is insufficient to induce pore complex disassembly), OA treatment induces immediate disassembly of NPCs and ALPCs (Figure 7C). Together these findings suggest a model where disassembly and reassembly of pore complexes during mitosis is regulated by a dynamic equilibrium of the antagonistic actions of Cdk1 and OA-sensitive phosphatases (Figure 9).

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