

An in vitro nuclear disassembly system reveals a role for the RanGTPase system and microtubule-dependent steps in nuclear envelope breakdown

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During prophase, vertebrate cells disassemble their nuclear envelope (NE) in the process of NE breakdown (NEBD). We have established an in vitro assay that uses mitotic *Xenopus laevis* egg extracts and semipermeabilized somatic cells bearing a green fluorescent protein–tagged NE marker to study the molecular requirements underlying the dynamic changes of the NE during NEBD by live microscopy. We applied our in vitro system to analyze the role of the Ran guanosine triphosphatase (GTPase) system in NEBD. Our study shows that

high levels of RanGTP affect the dynamics of late steps of NEBD in vitro. Also, inhibition of RanGTP production by RanT24N blocks the dynamic rupture of nuclei, suggesting that the local generation of RanGTP around chromatin may serve as a spatial cue in NEBD. Furthermore, the microtubule-depolymerizing drug nocodazole interferes with late steps of nuclear disassembly in vitro. High resolution live cell imaging reveals that microtubules are involved in the completion of NEBD in vivo by facilitating the efficient removal of membranes from chromatin.

Introduction

The nuclear envelope (NE) consists of an outer nuclear membrane and an inner nuclear membrane (INM). The outer nuclear membrane is continuous with the membrane system of the ER, whereas the INM contains a specific set of transmembrane proteins and is closely associated with the nuclear lamina and the chromatin. At sites where both membranes are fused, nuclear pore complexes (NPCs) are inserted, which serve the receptor-mediated exchange of macromolecules between the nucleus and the cytoplasm.

The small GTPase Ran plays a pivotal role in determining the directionality of nuclear transport during interphase of the cell cycle, but it is also used to mark the position and identity of chromatin during mitosis. In interphase, Ran is enriched in the nucleus, where it is in its GTP-bound form as a result of the action of the chromatin-bound guanyl-nucleotide exchange factor RCC1. In the cytoplasm, RanGTP is readily converted to RanGDP by the RanGTPase-activating protein (RanGAP) that stimulates the GTPase activity of Ran. During mitosis, the generation of RanGTP around chromatin persists (Kalab et al., 2006), providing

spatial information for spindle formation and NE assembly (for reviews see Hetzer et al., 2002; Weis, 2003).

At the onset of mitosis, major structural reorganizations of the cell occur, including NE breakdown (NEBD), condensation of chromosomes, and formation of a mitotic spindle. NEBD involves the disassembly of the NPCs, the depolymerization and solubilization of the lamina, and the detachment and removal of the nuclear membrane from chromatin, resulting in the redistribution of NE membrane proteins to the ER network (Ellenberg et al., 1997; Terasaki, 2000).

NEBD is a phosphorylation-dependent process. Phosphorylation of NE components is thought to disrupt the protein–protein interactions required for nuclear integrity. Several kinases have been implicated in the nuclear disassembly process, namely Cdk1–cyclin B, PKC (for review see Buendia et al., 2001), NIMA (never in mitosis A; Wu et al., 1998; De Souza et al., 2003), Cdk–cyclin A2 (Gong et al., 2007), and others (Miller et al., 1999). The activation of Cdk1–cyclin B leads to the mitotic hyperphosphorylation of lamins, resulting in the depolymerization of higher order lamin polymers and solubilization of the lamin proteins (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Heald and McKeon, 1990; Peter et al., 1990). Besides Cdk1–cyclin B, PKC is required for NEBD, and the PKC isoform PKC β II phosphorylates lamin B (Goss et al., 1994; Thompson and Fields, 1996; Collas, 1999).

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Abbreviations used in this paper: CSF, cytosolic factor; IBB, importin β -binding domain; INM, inner nuclear membrane; LAP2 β , lamina-associated polypeptide 2 β ; NE, nuclear envelope; NEBD, NE breakdown; NPC, nuclear pore complex; PB, permeabilization buffer; RanGAP, RanGTPase-activating protein.

The online version of this article contains supplemental material.

Other constituents of the NE are also targets for mitotic phosphorylation, including INM proteins (Courvalin et al., 1992; Foisner and Gerace, 1993; Ellis et al., 1998; Dreger et al., 1999) and nucleoporins (Macaulay et al., 1995; Favreau et al., 1996; Miller et al., 1999; De Souza et al., 2004), which are the constituents of the NPC. Interestingly, nucleoporins might be involved in NEBD beyond being phosphorylation substrates. Certain nucleoporins have been suggested to serve as landing pads for the COPI (coat protein I) coatamer complex, which might assist NE disassembly in a yet to be defined mechanism (Liu et al., 2003; Prunuske et al., 2006).

Studies in *Drosophila melanogaster* embryos and starfish oocytes suggest that NPC disassembly is the initial step of NEBD (Kiseleva et al., 2001; Terasaki et al., 2001; Lénart and Ellenberg, 2003). When the relative timing of NPC disassembly, NE rupture, and lamina solubilization was investigated in starfish oocytes, two phases of NE permeabilization were observed. During the first phase of NEBD, NPCs became partially dismantled, allowing the influx of a 70-kD fluorescent dextran. The NE structure, including the lamina, remained intact during this first phase. Complete permeabilization of the NE during phase two resulted in a fenestration of the membrane (detected by the influx of 500-kD dextran) followed by the complete disassembly of the lamina. Fenestration is thought to represent the complete removal of the NPCs (Lénart et al., 2003). This two-step process is explained by the initial phosphorylation of nucleoporins facilitating partial NPC disassembly. The increasing NPC permeability would then allow kinases to enter the nucleus, to phosphorylate their targets, and to trigger lamina and final NPC disassembly.

In addition to the mitotic phosphorylation of NE components, a microtubule-based tearing process assists NE disassembly in somatic cells (Beaudouin et al., 2002; Salina et al., 2002). Dynein, which is recruited to the NE at prophase (Busson et al., 1998; Gonczy et al., 1999; Robinson et al., 1999), interacts with spindle microtubules, thereby creating tension on the NE, which finally leads to its rupture (Beaudouin et al., 2002; Salina et al., 2002). Rupture starts with the formation of one to three holes in the NE, which then rapidly expand over the nuclear surface. Interference with microtubule function by microtubule-depolymerizing drugs does not inhibit but delays NEBD (Beaudouin et al., 2002; Salina et al., 2002).

Although a general description of the dynamic process of NEBD is starting to emerge, very little is known about the molecular machinery behind it. We have established a visual assay to study NEBD in vitro that allows for monitoring morphological changes of the NE in semipermeabilized somatic cells. To induce NEBD, we use mitotic *Xenopus laevis* egg extracts, which are amenable to biochemical treatments. Importantly, the use of fully activated mitotic extracts enables the dissection of NEBD independently of signaling events leading to mitotic entry in vivo. This in vitro assay allows for the molecular characterization of events leading to mitotic nuclear disassembly. Using this system, we have investigated the molecular requirements of mitotic nuclear breakdown. Our analysis uncovered an important role for the RanGTPase system in the final steps of nuclear disassembly and expands previous evidence for a supportive function of microtubules in NEBD (Beaudouin et al., 2002; Salina et al., 2002).

Results

A novel in vitro system to study nuclear disassembly

To study the molecular requirements of NEBD, we have established an in vitro system that allows for the observation of NEBD by 4D fluorescence microscopy. Our assay uses semipermeabilized HeLa cells derived from a cell line expressing a fusion of GFP to the transmembrane and lamin-binding domains of lamina-associated polypeptide 2 β (LAP2 β), a protein of the INM. GFP-LAP2 β -expressing HeLa cells grown on coverslips were semipermeabilized with digitonin. The nuclei were then incubated with *Xenopus* egg extracts either in an interphase or a mitotic state. Changes in the permeability of the NE were visualized by nuclear influx of a TRITC-labeled 155-kD dextran.

Nuclei incubated in interphase extract remained intact over the 45-min time course of the experiment (Fig. 1 and Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). Neither visible changes in the structure of the NE nor changes in its permeability occurred. The incubation of nuclei in cytosolic factor (CSF)-arrested *Xenopus* egg extracts induced nuclear disassembly. In 70–80% of the analyzed nuclei, NEBD started \sim 10–15 min after the addition of the mitotic extract. A first visible indication of nuclear disassembly was the influx of the TRITC-labeled 155-kD dextran accompanied by some shrinkage of the nuclei. This first phase of NEBD was followed by the formation and expansion of holes such that roughly 40% of the nuclei contained holes 25–35 min after the addition of mitotic extract. At this time point, all nuclei were permeable for the fluorescent dextran. Complete nuclear breakdown, which is characterized by a dynamic rupture of the NE, occurred \sim 35–45 min after addition of the CSF extract (Fig. 1 and Video 2). Variations of the kinetics of the disassembly process were observed depending on the quality of the extracts and the condition of the cells.

When we compared the relative timing of influx of two differently sized (70 or 155 kD) fluorescent dextrans in the first 20 min of NEBD (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>), we noticed that both dextrans entered the nuclei with almost identical kinetics, with the entry of the 155-kD dextran being delayed by only \sim 1 min. The influx of both dextrans was rapid, whereas the loss of the GFP-tagged nucleoporins Nup58 and Nup98 from the nuclear rim appeared to be a gradual process, suggesting that the change in NE permeability does not require the disassembly of all copies of these nucleoporins at once (Fig. S1). Because of the almost identical kinetics of the influx of both dextrans during the disassembly reaction, we decided to use the 155-kD species to monitor changes in NE permeability in all further experiments.

Kinase dependence of NEBD

Two kinases have been directly implicated in NEBD, namely Cdk1-cyclin B1 and PKC β II (Heald and McKeon, 1990; Peter et al., 1990; Goss et al., 1994; Macaulay et al., 1995; Favreau et al., 1996; Collas, 1999; Gong et al., 2007). To verify that our system truly recapitulates NEBD, we tested whether NEBD in vitro was dependent on Cdk1 and PKC by using specific

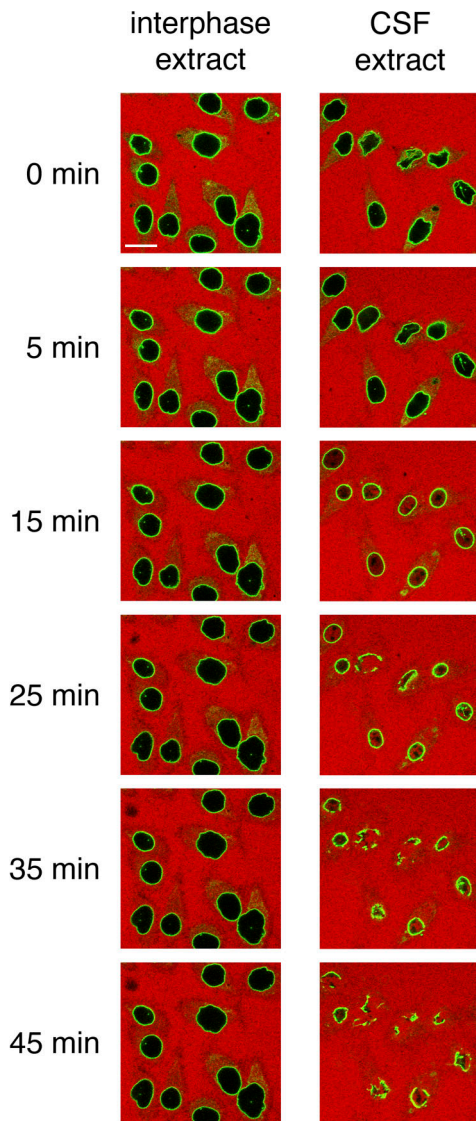


Figure 1. Incubation of HeLa cell nuclei in mitotic egg extracts results in nuclear disassembly. Time course of in vitro nuclear disassembly. HeLa cells stably expressing GFP-LAP2 β (green) were grown on coverslips to ~50% confluency. The cells were permeabilized and incubated in interphase (left) or mitotic (CSF; right) *Xenopus* egg extract. Extracts were supplemented with an energy-regenerating system and 250 μ g/ml of 155-kD TRITC-labeled dextran (red) to allow for the monitoring of changes in NE permeability. Nuclear disassembly was followed by time-lapse confocal laser-scanning microscopy. Bar, 20 μ m.

inhibitors of these kinases. Alsterpaullone was chosen as a Cdk1 inhibitor (Schultz et al., 1999). Gö6983 was used as a PKC inhibitor, as it inhibits several PKC isoforms, including PKC β (Gschwendt et al., 1996).

Inhibition of Cdk1 by treatment of the CSF extract with alsterpaullone strongly delayed the initiation of NEBD. Permeabilization of the nuclei started with the influx of dextran after ~20–25 min. NEBD proceeded slowly, and nuclei with holes could be only rarely detected after 45 min. The complete disassembly of nuclei failed (Fig. 2 A and Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). Inhibition of PKC activity by the addition of Gö6983 also inhibited nuclear disassembly, but differently (Fig. 2 A). The initiation

of NEBD was not substantially delayed, as dextran started to enter the nuclei at the same time as in control cells, and the nuclei also shrunk. However, most nuclei failed to form holes and to fenestrate; none disassembled.

The activity and specificity of the inhibitors were tested in a histone H1 phosphorylation assay. The addition of the inhibitors to the CSF extract showed that histone H1 phosphorylation was strongly reduced by alsterpaullone (reduction by 80%) and only moderately by the PKC inhibitor Gö6983 (reduction by 35%), showing that Cdk1 is the main histone H1 phosphorylating activity in the extract (Fig. 2 B). As expected, phosphorylation of histone H1 by recombinant Cdk1–cyclin B1 was reduced upon treatment with the Cdk1 inhibitor alsterpaullone. The PKC inhibitor Gö6983 did not affect Cdk1 activity, but it strongly reduced the phosphorylation of histone H1 by PKC β II. In reverse, alsterpaullone had no effect on PKC β II activity (Fig. 2 C).

Requirement for nucleocytoplasmic transport

Distinct subcellular localizations of key mitotic regulators are crucial for the initiation and progression through mitosis. This includes the nuclear accumulation of Cdk1–cyclin B1 at prophase before nuclear disassembly, a process that has been suggested to be important for Cdk1 activation (Hagting et al., 1998; Jin et al., 1998). Cdk1 present in our mitotic extract is fully active, making the nuclear import of Cdk1 unnecessary for its activation. This enabled us to directly test whether there are other requirements for nuclear import in the in vitro NEBD process or whether NEBD can, in principle, start from the cytoplasmic side.

To block nuclear import, we used a dominant-negative mutant of the nuclear import receptor importin β , Imp β _{45–462}, which is known to block all active transport through the NPC by stably binding to nucleoporins (Kutay et al., 1997). Imp β _{45–462} reliably blocked nuclear transport over the timespan of the experiment when tested in interphase extracts on semipermeabilized cells (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). The addition of Imp β _{45–462} to the disassembly reaction did not affect the kinetics of the initiation of NEBD (Fig. 3 A and Video 4). The influx of TRITC-labeled 155-kD dextran occurred with normal kinetics. Dextran entered 100% of the nuclei, and hole formation occurred as usual in ~40% of the nuclei. Neither the formation nor the expansion of these holes was affected by the inhibition of nuclear transport. The only observed effect of Imp β _{45–462} addition was that some of the completely holey nuclei failed to undergo the final dynamic rupture process. This is supported by high magnification confocal sectioning of disassembled nuclei at the end points of these disassembly reactions (Fig. 3 B).

The RanGTPase system and NEBD

To verify that nuclear import is not required for the onset of NEBD in the presence of fully activated mitotic extracts, we next tested the effect of RanQ69L addition. This GTPase-deficient Ran mutant stays GTP bound in the presence of RanGAP in the extracts, causing the dissociation of import cargoes from nuclear import receptors and, thereby, preventing the nuclear import of

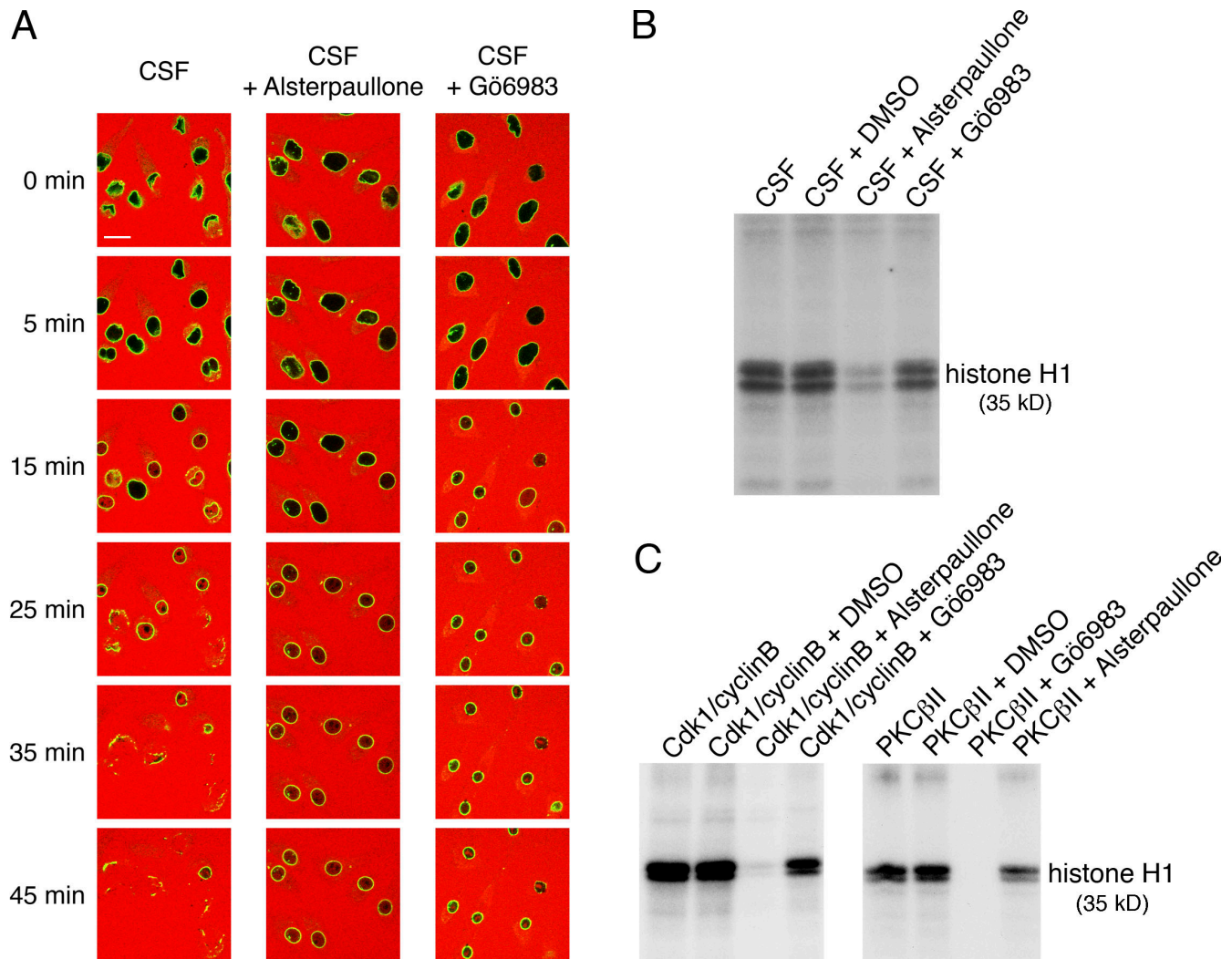


Figure 2. Efficient in vitro nuclear disassembly depends on Cdk1 and PKC activity. (A) Permeabilized GFP-LAP2 β (green)-expressing HeLa cells were incubated in CSF extracts containing an energy-regenerating system and a 155-kD TRITC-labeled dextran (red). Extracts were either untreated (solvent control; left) or treated with 200 μ M of the kinase inhibitors alsterpaullone (Cdk1 inhibitor; middle) or Gö6983 (PKC inhibitor; right) for 10 min at RT. Nuclear disassembly was monitored by time-lapse confocal laser-scanning microscopy. The relative fluorescence intensity inside nuclei ($n > 18$) was quantified, and an increase of $>10\%$ inside nuclei was used as a threshold. Dextran influx into nuclei incubated in untreated CSF extract and alsterpaullone-treated or Gö6983-treated extracts occurred after 15 min (± 4 min) and 22 min (± 3 min) or 12 min (± 2 min), respectively. (B) Kinase activity of untreated and inhibitor-treated CSF extracts was assayed using histone H1 as substrate. Final concentrations of alsterpaullone and Gö6983 were 200 μ M. (C) The specificity of the inhibitors used was tested using recombinant Cdk1–cyclin B1 and PKC β II. Bar, 20 μ m.

most nuclear proteins (Görlich et al., 1996b; Weis et al., 1996a). The addition of 20 μ M RanQ69L(GTP) to the CSF extract did not block the initial steps of nuclear disassembly (Fig. 4 A). The timing of dextran influx was unchanged. Compared with the control experiments, fewer nuclei formed holes, and, interestingly, these holes remained static and hardly expanded over the nuclear surface (Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). The final dynamic disassembly of the nuclei was blocked, as none of the nuclei disassembled after 45 min, underscoring this undynamic behavior. This RanQ69L(GTP)-mediated strong delay of NEBD is likely not caused by the inhibition of nuclear import because the initial kinetics of NE permeabilization was unchanged. NPC disassembly started normally, as shown by the unchanged kinetics of Nup58 dissociation from the nuclear rim (Fig. S3). RanQ69L(GTP) most

probably affects a later, dynamic step of nuclear breakdown, which normally facilitates the expansion of holes and, thus, disassembly. The phenotype of inhibition by RanQ69L(GTP) was different to treatment of the extracts with Imp β_{45-462} , which did not block complete disassembly. Disassembly in the presence of RanQ69L(GTP) was delayed at an earlier point, resulting in nuclei with fewer holes (Fig. 4 B).

The RanQ69L(GTP) effect could indicate that the GTPase activity of Ran is required for NEBD or, alternatively, that unbalancing the RanGDP/RanGTP ratio in the extract might cause disassembly defects. When endogenous RanGDP was converted to RanGTP by adding an excess of recombinant RCC1 to the extracts (Fig. 4, A and B; and Video 6, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>), NEBD was inhibited at the same stage as observed upon RanQ69L(GTP) addition.

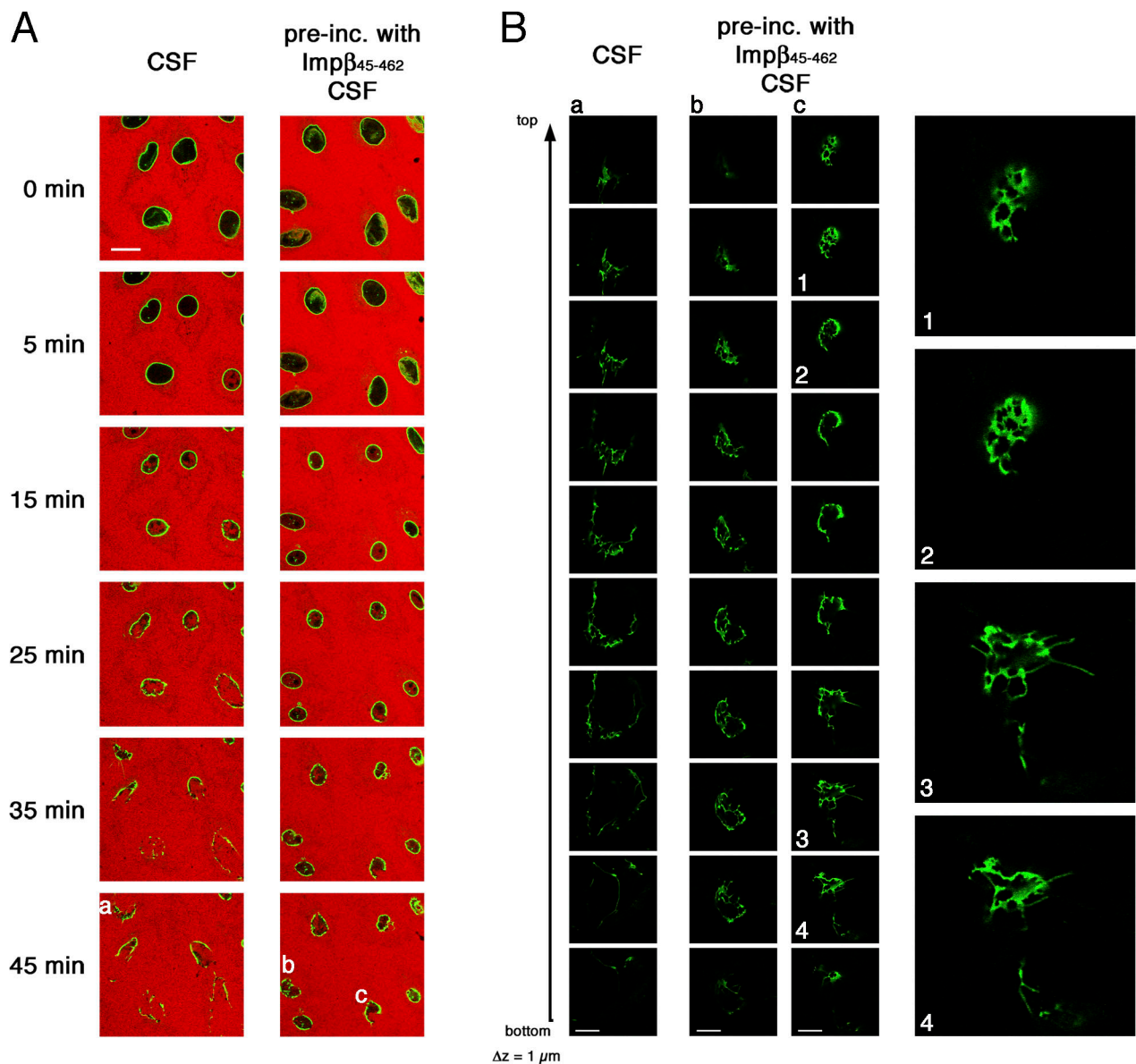


Figure 3. **Nuclear import is not required for the initiation of NEBD.** (A) To block nucleocytoplasmic transport, permeabilized GFP-LAP2 β (green)-expressing HeLa cells were preincubated with 15 μ M of recombinant Imp β_{45-462} (right). Nuclei were then incubated in CSF extracts supplemented with an energy-regenerating system and a 1.55-kD TRITC-labeled dextran (red). In vitro nuclear breakdown was monitored by time-lapse confocal laser-scanning microscopy. The relative fluorescence intensity inside nuclei ($n > 18$) was quantified as in Fig. 2. Dextran influx into nuclei of control cells or cells preincubated with Imp β_{45-462} occurred after 12 min (± 3 min) and 11 min (± 4 min), respectively. (B) High magnification confocal sectioning of disassembled nuclei from A (indicated by letters) was performed using a 63 \times objective and a 4 \times zoom. Z-step width is 1 μ m. On the right side, representative magnifications of selected frames from the z stacks (indicated by numbers) are shown. Bars (A), 20 μ m; (B) 10 μ m.

This indicates that a high RanGTP concentration in the egg extract affects NEBD and that proper function of the RanGTPase system might be required for NE disassembly.

Inhibition of directed nuclear transport itself does not seem to be responsible for the RanGTP-mediated disassembly defect. However, RanGTP might influence NEBD via the characteristic feature of nuclear transport receptors to bind or release their cargo in a RanGTP-dependent manner. For instance, RanQ69L(GTP) could stimulate export factors to sequester a component required for NEBD. A simple explanation for the effect of RanQ69L(GTP) or RCC1 could thus be that their addition alters the activity of Cdk1-cyclin B in the extracts

by driving the kinase heterodimer into a potentially inactive complex with an exportin (for instance, with CRM1; Hagting et al., 1998; Yang et al., 1998). Therefore, we analyzed the mitotic kinase activity of egg extracts in the presence of RanQ69L(GTP) or RCC1. The histone H1 phosphorylation assay shows that mitotic kinase activity of the extracts is not influenced by these treatments (Fig. 4 C).

Alternatively, the RanGTP effect on NEBD might be explained by a requirement for importins, which could aid nuclear disassembly by sequestering nuclear (envelope) components in the mitotic cytosol, a process prevented by RanQ69L(GTP). To address the involvement of importins, we used known competitors

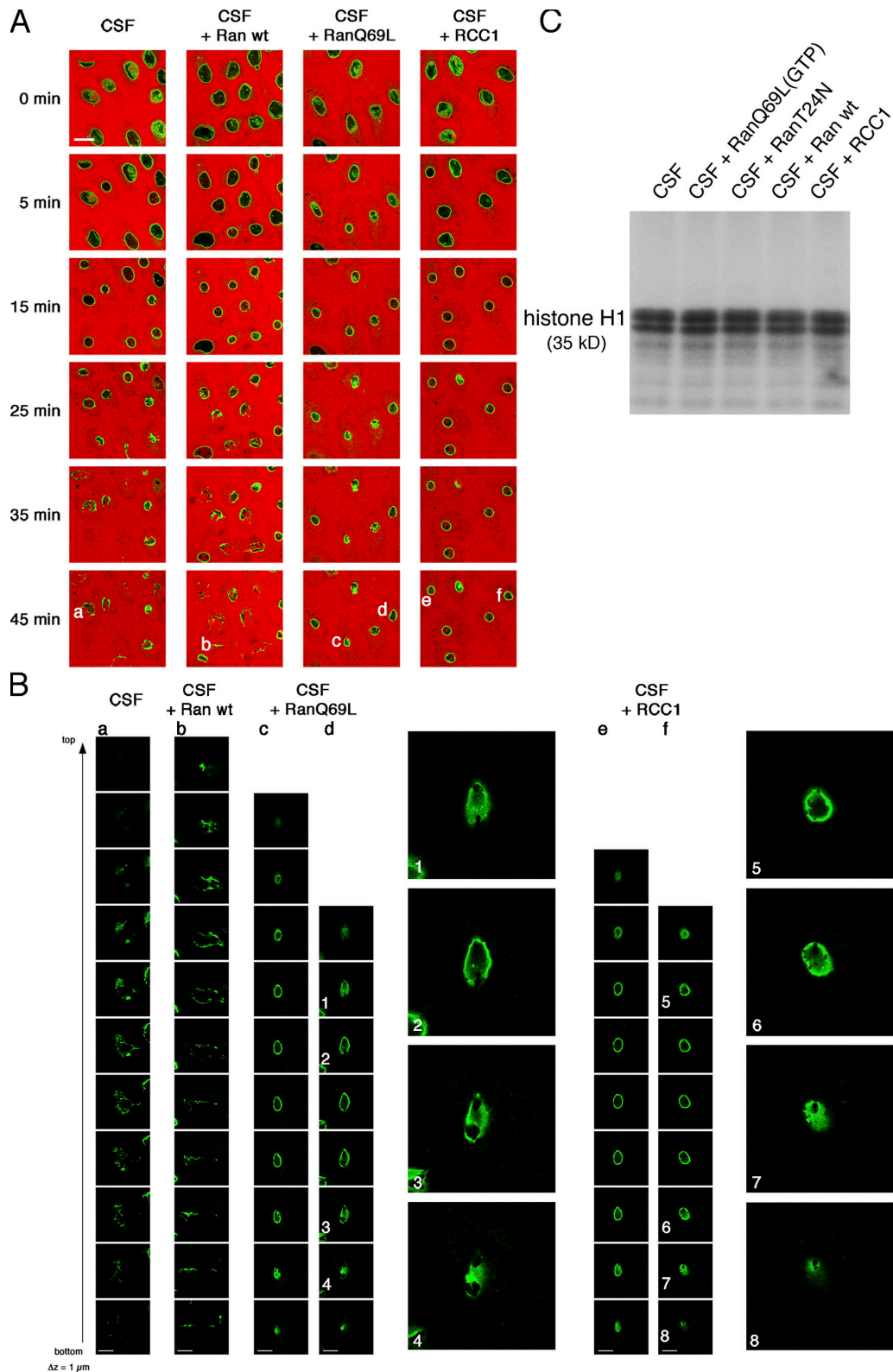


Figure 4. **Inhibition of NE rupture in vitro by excess of RanGTP in CSF extracts.** (A) Permeabilized GFP-LAP2 β (green)-expressing HeLa cells were incubated in untreated CSF extract or CSF extracts supplemented with 20 μ M of either recombinant Ran wild type (wt), recombinant RanQ69L(GTP), or RCC1. CSF extracts had been preincubated with the recombinant proteins for 10 min at RT before the disassembly reaction. Nuclear breakdown was monitored in the presence of 155-kD TRITC-labeled dextran (red) by time-lapse confocal laser-scanning microscopy. The relative fluorescence intensity inside nuclei ($n > 18$) was quantified as in Fig. 2. Dextran influx into nuclei incubated in untreated CSF extract or extracts incubated with Ran wild type, RanQ69L(GTP), or RCC1 occurred after 13 min (± 4 min), 11 min (± 5 min), 11 min (± 4 min), or 15 min (± 3 min), respectively. (B) High magnification confocal sectioning of disassembled nuclei from A (indicated by letters) was performed using a 63 \times objective and a 4 \times zoom. Z-step width is 1 μ m. Representative magnifications of selected frames from the z stacks (indicated by numbers) are shown for RanQ69L and RCC1 addition. (C) Kinase activity of untreated CSF extract and CSF extract supplemented with 20 μ M of recombinant RanQ69L(GTP), RanT24N, Ran wild type, or RCC1 was assayed using histone H1 as substrate. Bars (A), 20 μ m; (B) 10 μ m.

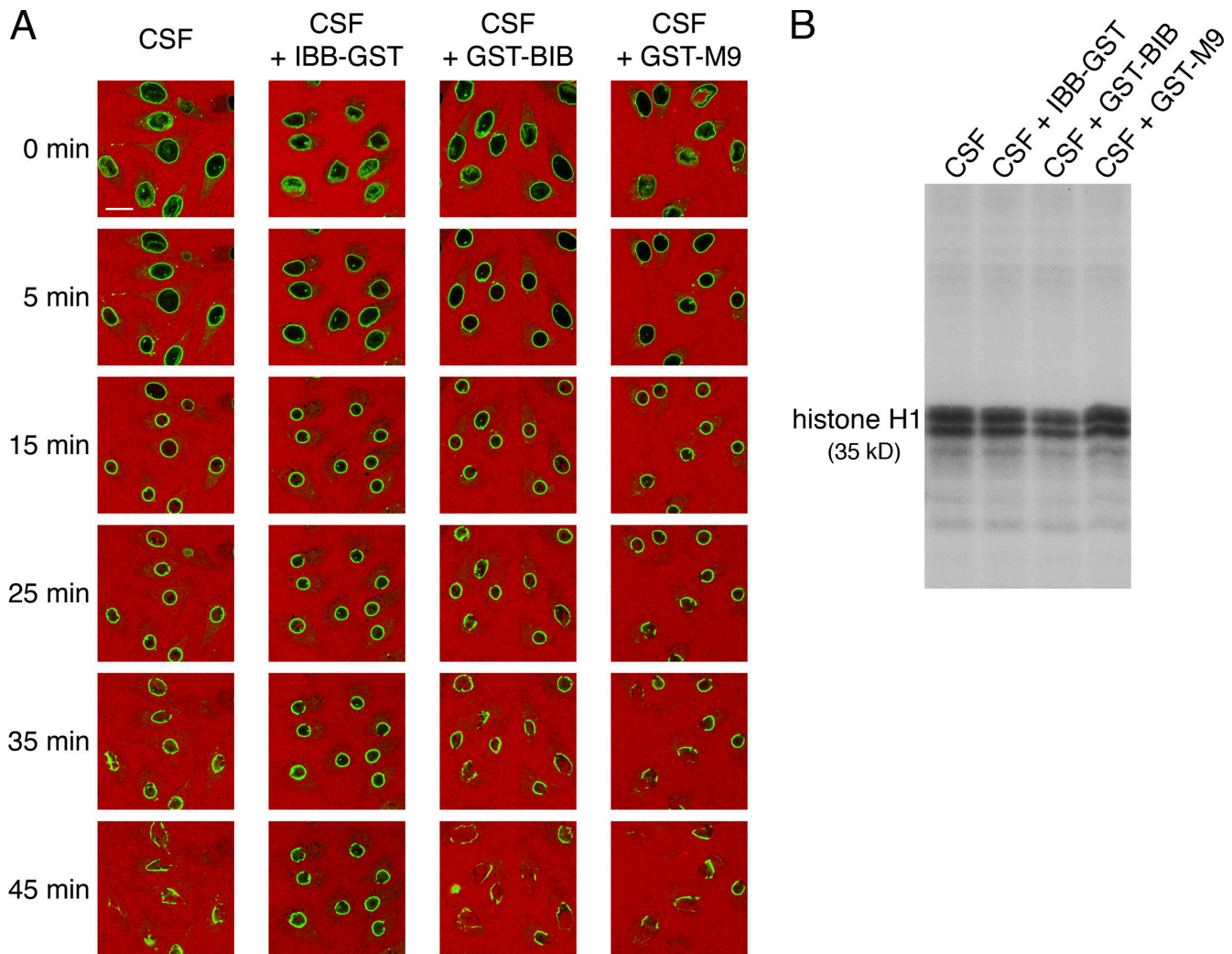


Figure 5. **The IBB of importin α inhibits the late steps of NEBD in vitro.** (A) Permeabilized GFP-LAP2 β (green)-expressing HeLa cells were incubated in untreated CSF extract (left) or CSF extract supplemented with recombinant IBB-GST, GST-BIB, or GST-M9 (20 μ M each; preincubated in the extract for 10 min at RT before the disassembly reaction). Nuclear disassembly was monitored in the presence of 155-kD TRITC-labeled dextran (red) by time-lapse confocal microscopy. (B) Kinase activity of untreated CSF extract and CSF extract supplemented with 20 μ M recombinant IBB-GST, GST-BIB, or GST-M9 was assayed using histone H1 as substrate. Bar, 20 μ m.

of cargo binding to different import receptors (Fig. 5 A). Strikingly, addition of the importin β -binding domain (IBB) of the NLS import adaptor importin α (Görlich et al., 1996a; Weis et al., 1996b) caused a similar block in NEBD as did RanGTP. NE permeabilization, as judged by the influx of fluorescent dextran, was normal, but NE fenestration and dynamic rupture were blocked. In contrast, the addition of two other nuclear transport competitors of distinct specificity, M9 (Siomi and Dreyfuss, 1995) and BIB (Jakel and Görlich, 1998), had no effect on NEBD. The effect of IBB was not the result of the inhibition of mitotic kinase activity in the extracts (Fig. 5 B). Many of the mitotic roles of the RanGTPase system are known to impinge on importin β (for review see Harel and Forbes, 2004), and our new data suggest that importin β 's capacity to associate with cargo is also required during late steps of NEBD. We further found that this role of importin β involves the binding of cargo to the NLS adaptor importin α , as NEBD was also impaired by a high concentration of a BSA-NLS

conjugate (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>).

The production of RanGTP normally occurs in the neighborhood of chromatin, where the RanGEF RCC1 is localized. RanGTP production around mitotic chromatin provides the positional information used for the local release of cofactors from inhibitory complexes with importins to allow for spindle assembly and NE reformation after mitosis (for reviews see Hetzer et al., 2002; Weis, 2003). Likewise, we reasoned that the activation of a disassembly-promoting factor close to chromatin aided by RanGTP might assist NEBD. To prevent RanGTP production around chromatin, we used a Ran mutant (RanT24N), which strongly binds and inhibits RCC1 because of its low affinity for guanyl nucleotides (Dasso et al., 1994). To block RCC1 activity, nuclei of semi-permeabilized cells were preloaded with RanT24N. Then, mitotic extract supplemented with RanT24N was added, and NEBD was monitored by live microscopy. Strikingly, RanT24N did not block early steps of the nuclear disassembly process but blocked

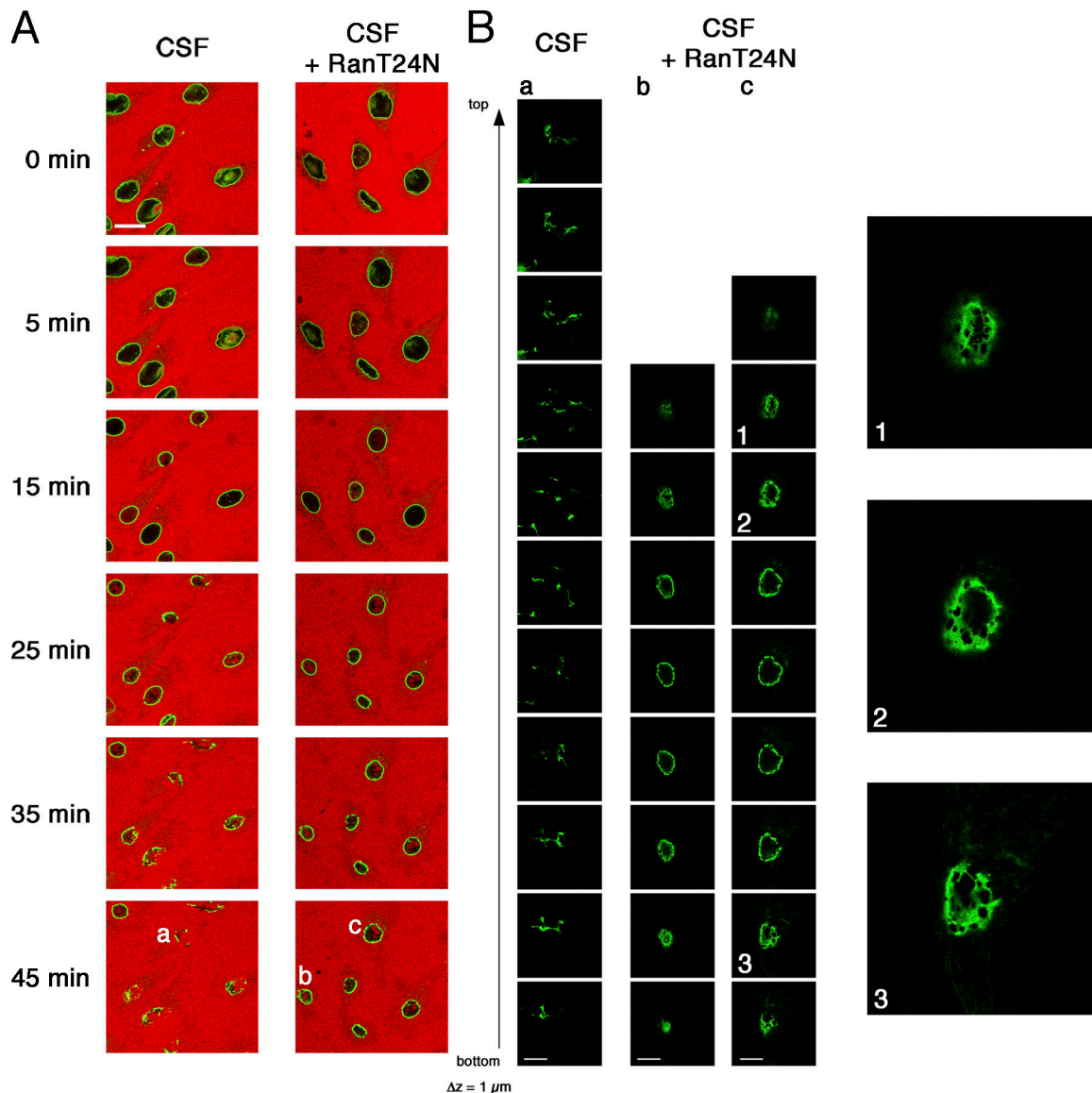


Figure 6. **Inhibition of RCC1 activity by RanT24N interferes with final steps of NEBD in vitro.** (A) Nuclei of semipermeabilized GFP-LAP2 β (green)-expressing HeLa cells were preloaded with either Ran wild-type (left) or RanT24N (right). Then, cells were incubated in CSF extract supplemented with either 20 μM Ran wild-type (left) or RanT24N (right), an energy-regenerating system, and a 155-kD TRITC-labeled dextran (red). NEBD was monitored by time-lapse confocal laser-scanning microscopy. The relative fluorescence intensity inside nuclei ($n > 14$) was quantified as in Fig. 2. Dextran influx into nuclei of control cells or cells preincubated with RanT24N occurred after 16 min (± 3 min) and 19 min (± 4 min), respectively. (B) High magnification confocal sectioning of disassembled nuclei from A (indicated by letters) was performed using a 63 \times objective and a 4 \times zoom. Z-step width is 1 μm . On the right, representative magnifications of selected frames from the z stacks (indicated by numbers) are shown. Bars (A), 20 μm ; (B) 10 μm .

the final dynamic NE rupture (Fig. 6 A and Video 7, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). Inspection of the nuclei at late time points revealed that nuclei were slightly perforated in the presence of RanT24N, indicating that NE disassembly had proceeded further than hole formation (Fig. 6 B). Control experiments showed that RanT24N was indeed blocking RCC1 activity, as Ran-dependent nuclear import was efficiently inhibited after preloading of nuclei with the Ran mutant (Fig. S2).

A microtubule-assisted step late in NEBD

The dynamic rupture of the nuclei in our in vitro disassembly assay hinted at a possible involvement of the microtubule

cytoskeleton. To test whether complete NEBD in vitro depended on forces exerted by microtubule-associated motor proteins, the CSF extract used for the disassembly reaction was treated with nocodazole. Early steps in NEBD were not affected by nocodazole, but, strikingly, the dynamic rupture of the nuclei was blocked (Fig. 7 A) even when the nuclei were incubated with the extract over time as long as 100 min (Video 8, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). Treatment with nocodazole had no influence on the activity of mitotic kinases in the extract, as shown by the unchanged ability of the extracts to phosphorylate histone H1 (Fig. 7 B).

Previous studies have suggested that a microtubule-based tearing mechanism supports the formation of holes in the NE at

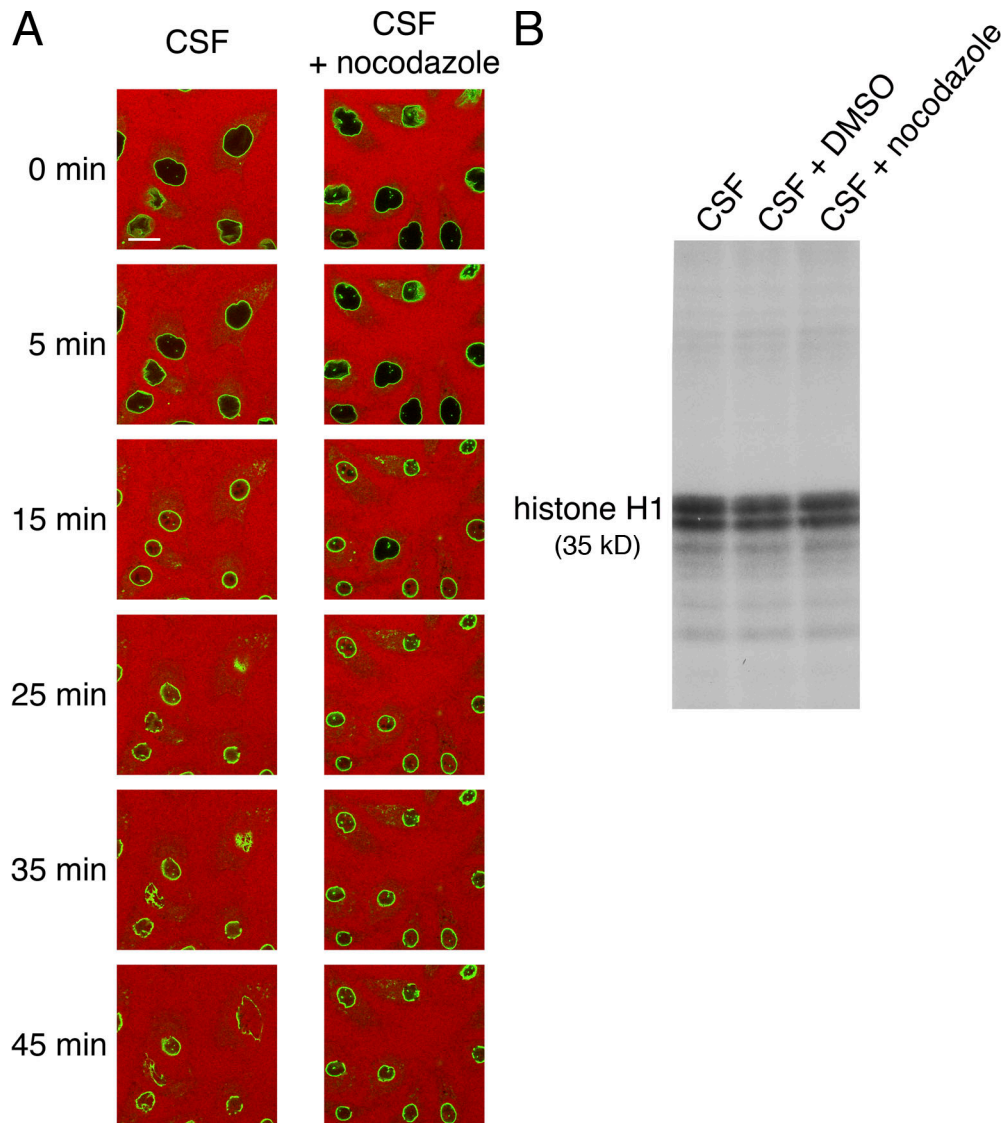


Figure 7. **Microtubules contribute to NE rupture in vitro.** (A) Permeabilized GFP-LAP2 β (green)-expressing HeLa cells were incubated in untreated CSF extract or CSF extract containing 10 μ M nocodazole (preincubated for 10 min at RT before the disassembly reaction). Nuclear disassembly was monitored in the presence of 155-kD TRITC-labeled dextran (red) by time-lapse confocal microscopy. (B) Kinase activity of untreated CSF extract and CSF extract supplemented with 10 μ M nocodazole, or the corresponding amount of DMSO was assayed using histone H1 as substrate. Bar, 20 μ m.

the onset of NEBD (Beaudouin et al., 2002; Salina et al., 2002). Our in vitro analysis indicated that microtubules might also aid a later step, namely the removal of NE membranes from the vicinity of chromatin. To gain in vivo evidence for our observation, we analyzed the fate of GFP-LAP2 β in nocodazole-treated HeLa cells in comparison with untreated cells. Time-lapse imaging revealed that in both cases, GFP-LAP2 β redistributed into tubular membrane structures at the onset of mitosis, which is consistent with the redistribution of INM proteins into the ER. Strikingly, in nocodazole-treated cells, the GFP-LAP2 β -containing membranes remained longer in close proximity to chromatin, which was visualized by H2B-mRFP (Videos 9 and 10, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). To gain a more quantitative insight into the effect of nocodazole on the timing of NE/ER removal from chromatin, we defined the starting point of NEBD by measuring changes in NE permeability

(Lénart and Ellenberg, 2006) using a mPlum-GST-M9 fusion, which localizes to the nucleus of interphase cells (as a result of constant nuclear import) and leaks out into the cytoplasm at the beginning of mitosis. 6, 10, and 14 min after the detection of mPlum-GST-M9 in the cytoplasm, marking the onset of NEBD, we took serial sections through cells (Fig. 8 A). Quantitative analysis of the images by measurement of the distances between the NE/ER membrane and chromatin supported the idea that the NE/ER network remained in the vicinity of the condensing chromatin for a longer timespan (Fig. 8 B). Whereas in untreated cells, membranes had been efficiently removed from the chromatin area, this process was significantly delayed in nocodazole-treated cells ($P < 0.01$). After 6 min, membrane removal was less efficient by at least a factor of six, and, 14 min after the initiation of NEBD, there was still a difference by a factor of two. Together, this analysis reveals that microtubules

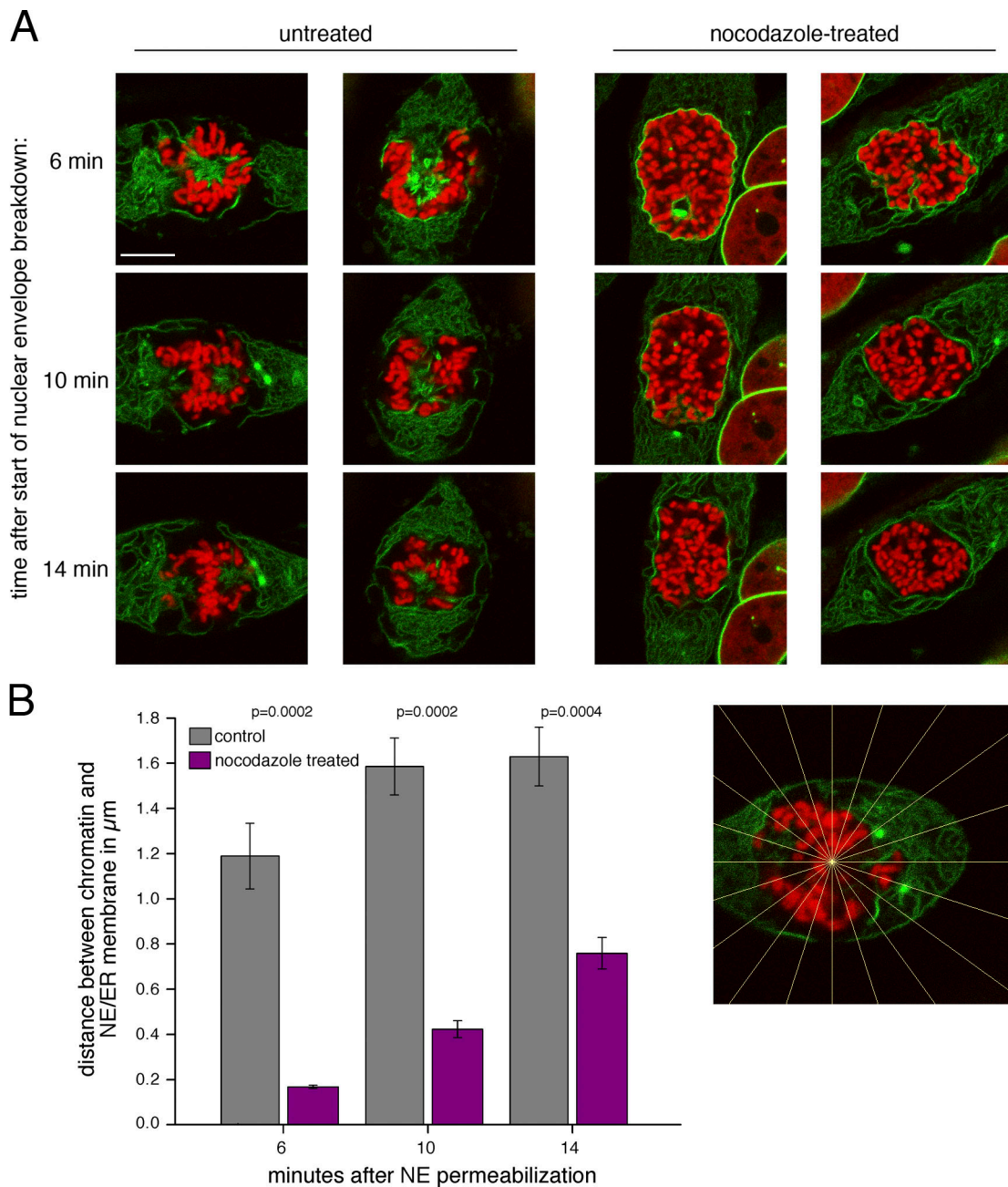


Figure 8. Microtubule-dependent removal of the NE/ER membrane network during mitosis in HeLa cells. (A) HeLa cells stably expressing GFP-LAP2 β (green) and H2B-mRFP (red) were transiently transfected with the plasmid coding for mPlum-GST-M9. Early prophase cells, which were either untreated or treated with 1 $\mu\text{g/ml}$ nocodazole, were chosen, and the efflux of mPlum-GST-M9 out of the nuclei was monitored by live confocal microscopy. The increase in NE permeability judged by a 1.3-fold increase of the fluorescence intensity of mPlum-GST-M9 in the cytoplasm (not depicted) was defined as the start of NEBD (time = 0 min). Stacks of four confocal images were obtained after 6, 10, and 14 min. (B) Distances between the chromatin and the NE/ER membrane were determined in the four slices of each stack along 20 lines of a radial grid (interval of lines = 18 $^\circ$) emanating from the center of the chromatin mass. Distances were determined for nine untreated cells and nine cells treated with nocodazole. Mean distances and SEM (error bars) are indicated. P-values were calculated using a directional Mann-Whitney test ($\alpha = 0.01$; $n = 9$). Bar, 10 μm .

play an important role in clearing the chromatin area from the NE/ER membrane network.

The microtubule minus end-directed motor protein dynein and its regulator dynactin localize to the NE late during G2 before any visible changes in mitotic NE organization occur (Salina et al., 2002). Dynein is thought to then promote the microtubule-dependent reorganization of the NE during prophase and NEBD. Overexpression of the dynactin subunits p62

or p50/dynamitin interferes with dynein function and was found to delay NEBD and to abolish NE invaginations during prophase, respectively (Salina et al., 2002). To test whether microtubule-dependent steps in NEBD *in vitro* are dependent on cytoplasmic dynein, we added recombinant p50/dynamitin to *in vitro* nuclear disassembly reactions. Strikingly, the dynamic disassembly of the nuclei was inhibited, whereas permeabilization and fenestration of the NE occurred with unchanged kinetics (Fig. S4).

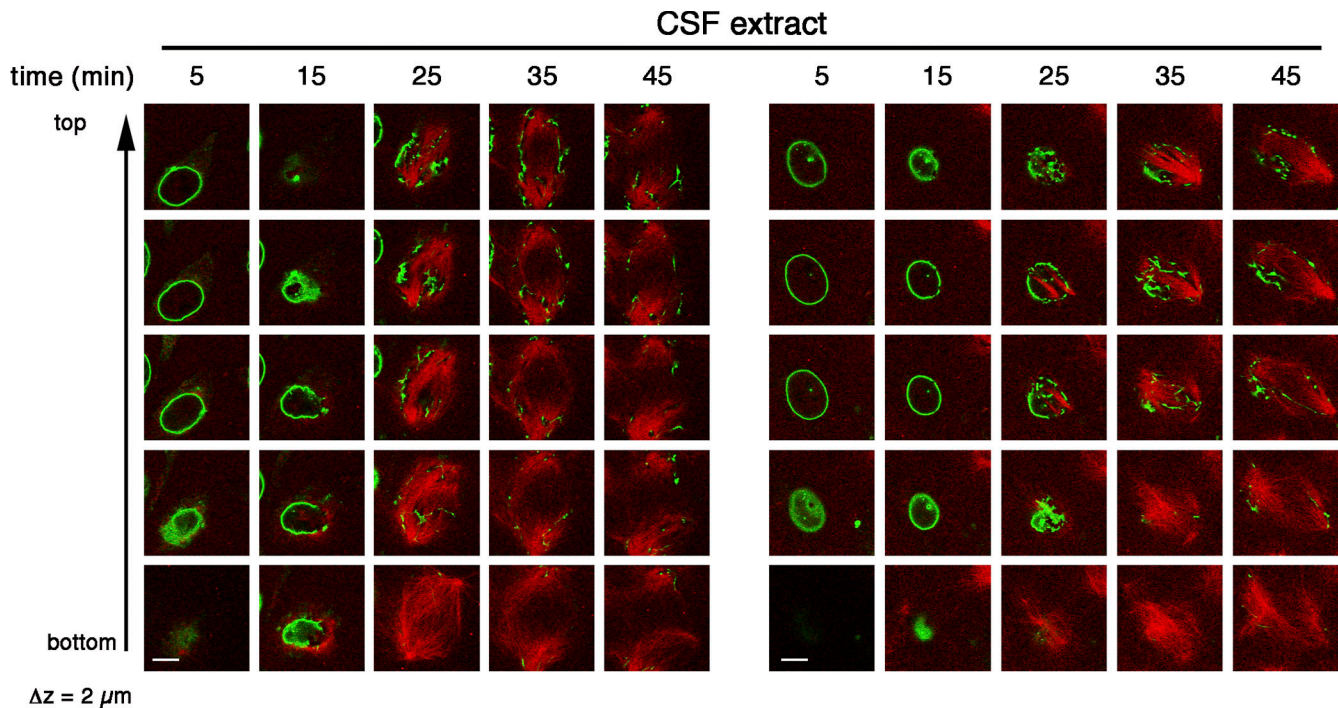


Figure 9. **Microtubule-dependent disintegration of the NE in vitro.** GFP-LAP2 β (green)-expressing HeLa cells were permeabilized and incubated with CSF extract supplemented with 2 μg rhodamine-labeled tubulin (red) and an energy-regenerating system. NEBD and microtubule polymerization were monitored by confocal laser-scanning microscopy. Five sections with a z-step width of 2 μm were taken at the indicated times. Bars, 10 μm .

Thus, the microtubule dependence of NEBD in vivo and in vitro appears to rely on the same molecular mechanism.

To visualize microtubule-based NE disintegration in vitro, we added rhodamine-labeled tubulin to the *Xenopus* egg extracts. The first visible sign of microtubule polymerization was seen in close proximity to the nuclei \sim 5–10 min after the addition of extract and was enhanced upon the formation of holes in the NE after 15 min (Fig. 9). Then, spindle-like structures formed at the position of the nuclear remnants. Subsequently, NE pieces were torn apart in association with microtubules, supporting the notion that the microtubule cytoskeleton directly aids the peripheral scattering of NE membranes.

Discussion

We have developed a visual assay to study NEBD in vitro that allows for monitoring morphological changes of the NE in semipermeabilized somatic cells. This in vitro assay provides a powerful tool to investigate the molecular mechanisms of NEBD. The progression of NEBD in vitro resembles the nuclear disassembly observed in vivo. Nuclear breakdown starts with permeabilization of the NE, which is followed by the formation of few holes, NE fenestration, and, finally, rupture of the nuclei. Also, on the molecular level, the in vitro system faithfully recapitulates many of the known requirements for NEBD.

First, in our system, early events of NEBD are dependent on Cdk1 and PKC activity. Cdk1 is the master regulator of mitotic entry, and it has long been difficult to uncouple its potential involvement in NEBD from its general requirement for progression into mitosis. Our system uses extracts containing

fully activated Cdk1. The requirement for Cdk1 activity to initiate NEBD in vitro implies a direct involvement of Cdk1 in the initiation of nuclear disassembly, likely by inducing NPC disassembly. This assumption is supported by the ability of Cdk1 to phosphorylate several nucleoporins in vitro (Macaulay et al., 1995). Interestingly, a recent study provided evidence for direct involvement of a Cdk–cyclin A complex in NEBD in vivo (Gong et al., 2007). The depletion of cyclin A2 from HeLa cells by RNAi did not affect cyclin B1–Cdk1 activation but delayed NEBD (Gong et al., 2007). However, it remains unclear whether cyclin A functions in NEBD in complex with Cdk1 or Cdk2.

Earlier studies had implicated PKC in mitotic entry. PKC β II is a well-characterized lamin B kinase (Goss et al., 1994; Thompson and Fields, 1996). Inhibition of PKC in synchronized human promyelocytic leukemia (HL60) cells leads to an arrest in G2 without inhibiting Cdk1 (Thompson and Fields, 1996), but it is currently unclear whether this G2 arrest is solely explained by the disturbed phosphorylation of NE components or whether events upstream of NEBD are affected.

Interestingly, our data revealed a differential requirement for Cdk1 and PKC activity in NEBD. The inhibition of Cdk1 delayed permeabilization of the NE and, therefore, is most probably important for NPC disassembly. However, PKC inhibition only mildly affected the kinetics of NE permeabilization, but all subsequent processes like hole formation and NE fenestration are blocked. Together, this indicates that Cdk1 might be required earlier during NEBD than PKC.

Second, consistent with in vivo studies analyzing NEBD in maturing starfish oocytes (Lénart and Ellenberg, 2003),

we provide evidence that NEBD can be initiated from the cytoplasmic side independently of nuclear transport. It has been proposed that regulation of the nucleocytoplasmic localization of protein kinases is crucial for initiation and progression through mitosis. Mitotic kinases such as Cdk1–cyclin B (Hagting et al., 1998, 1999) and PKC (Goss et al., 1994) accumulate in the nucleus before NEBD. The nuclear accumulation of Cdk1–cyclin B has been suggested to be critical for its complete activation as well as for triggering mitotic nuclear events (for review see Yang and Kornbluth, 1999). We used the dominant-negative mutant Imp β _{45–462} as an inhibitor of receptor-mediated nuclear transport and did not observe any delay in early events of NEBD. NE permeabilization and hole formation occurred as in control experiments, suggesting that in our in vitro system, in which mitotic kinases are fully active, NEBD can be initiated from the cytoplasmic side independently of nuclear transport. This is consistent with the notion that nuclear breakdown is initiated by nucleoporin phosphorylation, triggering NPC disassembly.

Third, we observed a strong dependence on microtubule dynamics for efficient nuclear disassembly in vitro. Previous in vivo studies demonstrated that microtubules are involved in the initial events of NEBD by mechanically supporting the formation of holes in the nuclear lamina and nuclear membrane (Beaudouin et al., 2002; Salina et al., 2002). In contrast to the in vivo situation, however, in which NEBD still occurs after the treatment of cells with nocodazole, NE rupture never occurred in the presence of this drug in our in vitro assay, even when NEBD was monitored for as long as 2 h. A reason for the strict dependence on microtubule-based tearing in our in vitro system might lie in a failure to retract INM proteins into the ER. We currently do not know why our system fails to reproduce this process, as the ER appears to be intact after cell permeabilization (not depicted).

Time-lapse microscopy of living cells had previously revealed that NEBD is already delayed at early steps like the initial hole formation of the NE when exposed to nocodazole (Beaudouin et al., 2002; Salina et al., 2002). In addition, microtubules may also aid subsequent steps of nuclear disintegration, as NE markers are still present in the vicinity of chromatin in prometaphase cells after nocodazole treatment, as suggested by the examination of fixed cells (Salina et al., 2002). Our in vivo analysis of the INM protein LAP2 β in cells entering mitosis extends these previous observations. We observed that the dispersal of GFP-LAP2 β into the ER in prophase occurs with similar dynamics as in untreated cells, whereas removal of the membrane from the chromatin area was strongly delayed by nocodazole. Therefore, it seems that microtubules are not only involved in the early steps of NEBD like hole formation but are also important during a later step of NEBD when the NE/ER network is pulled away from the chromatin toward the centrosomes.

Previous studies using nuclei that were first assembled in vitro in *Xenopus* egg extracts and dismantled in the presence of mitotic egg extracts have implicated the COPI coatomer complex and ADP-ribosylation factor (ARF) in NEBD (Liu et al., 2003). Analysis of NE disintegration in this experimental set-up by electron microscopy revealed ER-like membrane tubules and vesicles emanating from the NE (Cotter et al., 2007).

Membrane disassembly in this system is sensitive to brefeldin A and inhibited by ARF peptides. In our system, which uses nuclei of semipermeabilized somatic cells, we were unable to detect any inhibition of NEBD by brefeldin A or ARF peptides (unpublished data), indicating that the two experimental systems might differ. In living somatic cells, NE membranes retract back into the ER without any obvious sign of vesiculation (Ellenberg et al., 1997). Therefore, it remains to be seen whether there is a necessity for a vesiculation pathway during the disassembly of somatic cell nuclei in vivo.

One great advantage of the in vitro system lies in the opportunity to interfere with the function of components that are required throughout the cell cycle in living cells and to specifically investigate their function in NEBD. One such factor is RanGTP, which defines the identity of chromatin throughout the cell cycle. Indeed, our results show that the proper balance between RanGTP in the vicinity of chromatin and RanGDP in the mitotic extracts is required for late steps of NEBD in vitro but not for initial NE permeabilization, supporting the finding that nuclear import is not required for initial events in NEBD in this system.

The RanGTPase system is involved in other mitotic events like spindle assembly and NE reformation (for reviews see Hetzer et al., 2002; Suntharalingam and Wentz, 2003; Weis, 2003; Harel and Forbes, 2004). Studies on spindle formation using *Xenopus* egg extracts demonstrated that RanGTP induces the release of microtubule-associated proteins like TPX2 or NuMA from importins α and β , thereby stimulating microtubule assembly (Gruss et al., 2001; Nachury et al., 2001). Likewise, the release of nucleoporins from complexes with importin β by RanGTP in the vicinity of chromatin has been suggested to spatially regulate the recruitment of nucleoporins to chromatin at the end of mitosis (Harel et al., 2003; Walther et al., 2003).

Similarly, block of the final stages of in vitro NEBD induced by RanGTP or ectopic RCC1 might be ascribed to RanGTP's role in regulating the binding of cargo to nuclear transport receptors. As NEBD was inhibited by excess of the IBB of importin α in a similar way as by RanGTP, it is tempting to speculate that sequestration of NE components by importin β in the mitotic cytosol might facilitate late steps in NEBD. Both RanGTP and IBB would prevent such a function of importin β . Candidate NE components sequestered by importin β are nuclear lamins that carry classical nuclear localization signals (Loewinger and McKeon, 1988). Furthermore, several INM proteins contain NLS-like sequences, and it has recently been suggested that importins α and β are directly involved in binding to and escorting INM proteins across the NPC to the nuclear interior (King et al., 2006). Mitotic depolymerization of the nuclear lamina depends on the phosphorylation of lamins and lamin-binding proteins of the INM. However, efficient dissolving of chromatin-lamina-INM contacts might not only require the phosphorylation of structural components but also their sequestration in the mitotic cytosol by binding to importins, thereby preventing their tendency to repolymerize. Thus, high levels of RanGTP and IBB might interfere with the sequestration of these proteins; repolymerization might occur and result

in a block of dynamic rupture of the nuclear membrane. Strikingly, RanGTP has been reported to induce the polymerization of lamin-containing structures in mitotic *Xenopus* extracts in vitro (Tsai et al., 2006).

According to a different but not mutually exclusive scenario, RanQ69L(GTP) and IBB may exert their effects on microtubules that aid NE disintegration. High levels of RanGTP or the presence of IBB, which are both known to induce chromatin-independent microtubule aster formation in *Xenopus* egg extracts (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999), might hinder the normal attachment of microtubules to the NE. Notably, not only high levels of RanGTP but also RanT24N, which inhibits RanGTP production by RCC1, interfered with the final processes of NEBD. However, the phenotypes of RanT24N and RanQ69L(GTP) addition on NEBD differed in that RanT24N only inhibited the final dynamic rupture of NE, leaving the NE fully fenestrated, whereas RanQ69L(GTP) treatment essentially blocked NEBD after hole formation. Strikingly, both treatments affected the productive tearing of NE remnants along microtubules in bipolar spindlelike structures (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>). Whereas RanT24N addition reduced microtubule polymerization/stability, RanQ69L induced the formation of multiple asters around the holey nuclei, which, however, were nonproductive in exerting forces on the NE. Together, our observations can be reconciled by the existence of several distinct steps late during NEBD that differentially rely on the proper function of the RanGTPase system. Perhaps the transition from hole formation to fenestration requires the sequestration of NE components in the mitotic cytosol (inhibited by RanQ69L(GTP), ectopic RCC1, and IBB), whereas a subsequent step of microtubule-based NE tearing depends on the local production of RanGTP around chromatin, likely by aiding proper spindle assembly.

Clearly, the RanGTPase system also plays a pivotal role in mitotic entry in somatic cells in vivo, not only in controlling the nucleocytoplasmic distribution of mitotic regulators. Similar to the situation in our in vitro system, microinjection of RanQ69L(GTP) into HeLa prophase cells induces ectopic microtubule asters around the NE/chromatin (Kalab et al., 2006), but it has not yet been addressed whether RanQ69L(GTP) injection affects NE disintegration. Such in vivo, studies are complicated by the multiple roles of the RanGTPase system. Therefore, one would benefit from knowing the molecular targets of Ran action in NEBD. Exploiting the in vitro NEBD system will certainly help their identification in the future.

Materials and methods

DNA constructs

pcDNA3GFP-LAP2 β (aa 244–453) was a gift from T. Rapoport (Harvard Medical School, Boston, MA). mRFP-LAP2 β was generated by replacing EGFP with the mRFP coding sequence (Campbell et al., 2002) in the backbone of pEGFP-C3 (CLONTECH Laboratories, Inc.) and subsequently inserting the coding sequence of LAP2 β (aa 244–453) into the HindIII and BamHI sites. pEGFP $_2$ -Nup58 and pEGFP-Nup98 were gifts from J. Ellenberg (European Molecular Biology Laboratory, Heidelberg, Germany; Rabut et al., 2004). H2B-mRFP (Keppler et al., 2006) in a modified pRESpuro vector (CLONTECH Laboratories, Inc.) was a gift from D. Gerlich (Swiss Federal

Institute of Technology, Zurich, Switzerland). mPlum-GST-M9 was generated by replacing the coding sequence of EGFP in pEGFP-C1 (CLONTECH Laboratories, Inc.) by the coding sequence of mPlum (Wang et al., 2004) and ligating the coding sequence of the GST-M9 fusion protein into the BglII and HindIII sites. Plasmids used for the recombinant protein expression of Imp β _{45–462}, wild-type Ran, RanQ69L, RanT24N, RCC1, GST-M9, IBB-GST, and GST-BIB have been described previously (Mühlhäusser et al., 2001). The plasmid for the bacterial expression of p50/dynamitin was a gift from T. Hyman (Max Planck Institute, Dresden, Germany). For the expression of Cdk1–cyclin B1 in insect cells, the coding sequences of cyclin B1 (GenBank/EMBL/DDJB accession no. NM_031966) and Cdk1 (GenBank/EMBL/DDJB accession no. NM_033379) were amplified by PCR from HeLa cell cDNA and were cloned into the NcoI–HindIII and BamHI–HindIII sites, respectively, of the pFastBac HTb vector from the BAC-TO-BAC Baculovirus Expression System (Invitrogen). Transposition into the bacmid, transfection of Sf9 insect cells, and amplification of viral stocks were performed as described by the manufacturer.

Cell culture, cell lines, and transfections

Stable cell lines were obtained after the transfection of HeLa cells with the plasmid coding for GFP-LAP2 β followed by selection for positive clones with 500 μ g/ml G418. Subsequently, H2B-mRFP was introduced into this cell line, and double stable clones were selected with 500 μ g/ml G418 and 0.5 μ g/ml puromycin. Transfections were performed using FuGENE 6 (Roche). HeLa cells were maintained in complete DME (containing 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin) and the appropriate selection drugs. For in vivo imaging, cells were cultured in chambered coverglasses (LabTekII; Nunc) in complete DME without phenol red (Invitrogen) containing an additional 10% FCS.

Recombinant protein expression and purification

Expression and purification of wild-type Ran, RanQ69L, RanT24N, RCC1, Imp β _{45–462}, GST-M9, IBB-GST, and GST-BIB have been described previously (Mühlhäusser et al., 2001). Expression and purification of p50/dynamitin has been described previously (Wittmann and Hyman, 1999). Before addition to CSF extracts, the buffer of wild-type Ran, RanQ69L, RanT24N, RCC1, GST-M9, IBB-GST, and GST-BIB was exchanged to 50 mM Hepes, pH 7.4, 250 mM KoAc, and 2 mM MgOAc. The buffer of p50/dynamitin was exchanged to permeabilization buffer (PB).

Cdk1 and cyclin B1 were coexpressed in baculovirus-infected Sf21 cells. 3 d after infection, the cells were harvested, washed once, and resuspended in 1 vol of ice-cold hypotonic buffer (10 mM Hepes, pH 7.5, 25 mM NaCl, 0.5 mM EDTA, 10 μ g/ml cytochalasin B, 2 mM PMSF, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 5 mM NaF, and 1 mM Na₃VO₄). After Dounce homogenization, the NaCl concentration was adjusted to 150 mM, the lysate was cleared by centrifugation, and MgCl₂ was added to a final concentration of 5 mM. Cdk1–cyclin B1 was purified via Ni²⁺-NTA-agarose chromatography. After elution, protein-containing fractions were pooled and concentrated to a volume of 100–200 μ l. The Cdk1–cyclin B1 complex was separated from monomeric Cdk1 and cyclin B1 by gel filtration using a Superdex 200 HR 10/30 column (GE Healthcare) in 25 mM Hepes, pH 7.5, 150 mM NaCl, 10% (wt/vol) glycerol, and 1 mM DTT. PKC β II was purchased from Panvera.

Inhibitors

Alsterpaullone and Gö6983 were purchased from Calbiochem. Nocodazole was purchased from Sigma-Aldrich.

Xenopus egg extract preparation

Priming of *Xenopus* for ovulation and preparation of interphase and CSF-arrested (mitotic) egg extracts were performed essentially as described previously (Murray, 1991). Eggs were washed once in 2% cysteine, pH 8.0, dejellied for 5 min in 2% cysteine, pH 8.0, and washed three times in 1 mM Hepes, pH 7.8, 20 mM NaCl, 0.4 mM KCl, 0.2 mM MgSO₄, 0.5 mM CaCl₂, and 16 μ M EDTA. For interphase egg extract preparation, eggs were washed three times in 10 mM Hepes, pH 7.7, 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, and 250 mM sucrose. For CSF extract preparation, eggs were washed three times in 10 mM Hepes, pH 7.7, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 50 mM sucrose, and washed three times in the same solution additionally containing 1 mM MgCl₂ and 5 mM EGTA. After washing, the eggs were transferred into centrifuge tubes (model 331372; Beckman Coulter), packed by spinning at 400 g for 5 min in a SW41 rotor (Beckman Coulter), and the extra buffer was removed from the top of the eggs. Aprotinin, leupeptin, and cytochalasin B were added to a final concentration of 5 μ g/ml each, and caspase inhibitors (caspase-3 inhibitor I and caspase inhibitor II; Calbiochem) were added to a final

concentration of 20 nM. Eggs were crushed by spinning at 12,000 g for 20 min, and crude extracts were harvested from the centrifuge tubes using a 19-gauge needle. The protein concentration of the crude extracts is ~30 mg/ml. Crude extracts were supplemented with 250 mM sucrose and stored in small aliquots at -80°C. After thawing, an energy-regenerating system was added to 0.25 mM GTP, 0.25 mM ATP, 5 mM creatine phosphate, and 25 µg/ml creatine kinase.

In vitro nuclear disassembly reaction

Evenly spread HeLa cells grown on coverslips were permeabilized in PB (20 mM Hepes, pH 7.4, 110 mM KOAc, 5 mM MgOAc, 0.5 mM EGTA, and 250 mM sucrose) containing 40 µg/ml digitonin for 10 min at RT. The cells were then washed three times in PB for 2, 5, and 8 min and used for the disassembly reaction within the next 10 min. The coverslips were mounted on chambered slides together with 20 µl of egg extract. Where indicated, 250 µg/ml of 155-kD TRITC-labeled dextran or 70-kD Alexa-Fluor647-labeled dextran was added.

Preloading of HeLa cell nuclei with Ran or RanT24N was performed by incubation of permeabilized cells for 7 min with PB containing 7.5 µM RanT24N, 1.5 µM NTF2, 0.75 µM RanBP1, and 0.5 µM RanGAP. Preincubation of permeabilized nuclei with Impβ₄₅₋₄₆₂ was performed for 7 min with PB containing 15 µM Impβ₄₅₋₄₆₂. Note that all control disassembly reactions contained a buffer control with equal amounts of solvents or buffers that have been used for the addition of inhibitors or recombinant proteins.

Kinase assay

1 µl of egg extract was incubated with 9 µl H1 kinase buffer (80 mM β-glycerophosphate, pH 7.4, 15 mM MgCl₂, 20 mM EGTA, 5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 mM NaF, 1 mM Na₃VO₄, and 0.1 mM ATP) containing 3 µg histone H1 and 2 µCi γ-[³²P]ATP. To assay the kinase activity of recombinant kinases, 0.5 µg of purified Cdk1-cyclin B1 and 0.04 µg PKCβII were incubated with 9 µl H1 kinase buffer containing 3 µg histone H1 and 2 µCi γ-[³²P]ATP. The kinase reaction was performed for 10 min at 30°C and stopped by the addition of 30 µl SDS sample buffer. One fourth of each reaction was run on a 14% polyacrylamide gel.

Image acquisition and processing

For confocal laser-scanning microscopy of in vitro disassembly reactions, a 63× 1.4 NA differential interference contrast plan Aplanachromat oil immersion objective (Carl Zeiss MicroImaging, Inc.) mounted on a microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) and a confocal scanning module (excitation at 488 and 543 nm; LSM510 META; Carl Zeiss MicroImaging, Inc.) were used. In 5-min intervals, confocal sections of selected areas were captured using macros allowing multiposition time-lapse image acquisition and image file concatenation (Rabut and Ellenberg, 2004). Live cell imaging was performed using a 63× 1.4 NA differential interference contrast plan Aplanachromat oil immersion objective (Carl Zeiss MicroImaging, Inc.) mounted on a customized confocal microscope (excitation 488 and 561 nm; LSM510; Carl Zeiss MicroImaging, Inc.) equipped with a temperature- and CO₂-controlled incubator box. To monitor NEBD of individual cells with high time resolution, image stacks of 512 × 512 × 7 with a width of 35.7 µm and a 1-µm step size were acquired every 15 s (pixel time of 0.80 µs; line average 1). For subsequent measurements of distances between chromatin and NE/ER membranes, stacks of 512 × 512 × 4 with a width of 35.7 µm and a step size of 1 µm were acquired at the indicated times (pixel time of 0.80 µs; line average 8). Images were further analyzed and processed using LSM software (Carl Zeiss MicroImaging, Inc.) and ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>). Distances between the chromatin and the NE/ER membrane system were determined using the ImageJ software and a customized version of the radial grid plug-in. A radial grid of 20 lines (angles between lines were 18°) was placed in the center of the chromatin mass. In all four slices of a stack, the distance between the chromatin and the NE/ER membrane was manually determined along the lines of the grid.

Online supplemental material

Videos 1-7 show time-lapse videos corresponding to the experiments presented in Figs. 1 (Video 1, interphase extract; Video 2, CSF extract), 2 A (Video 3, Cdk1 inhibition), 3 (Video 4, Impβ₄₅₋₄₆₂ addition), 4 (Video 5, RanQ69L addition; Video 6, RCC1 addition), and 6 (Video 7, RanT24N addition). Video 8 shows a time-lapse video of NEBD in vitro in the presence of nocodazole over a time of 100 min. Videos 9 and 10 compare the progression of GFP-LAP2β-expressing HeLa cells into mitosis in the absence or presence of nocodazole, respectively. Fig. S1 shows the time

course of the nuclear influx of 70- and 155-kD dextrans as well as the kinetics of nucleoporin disassembly using GFP₂-Nup58 and GFP-Nup98 as markers. Fig. S2 demonstrates that nuclear accumulation of a fluorescently labeled NLS-containing substrate is blocked by Impβ₄₅₋₄₆₂, RanQ69L(GTP), and RanT24N, inhibited by RCC1, and competed by unlabeled BSA-NLS conjugates. Fig. S3 shows that the addition of RanQ69L does not interfere with the initial steps of NPC disassembly. Fig. S4 provides evidence that late steps of NEBD in vitro are inhibited by the addition of BSA-NLS conjugates or by recombinant p50/dynamin. In Fig. S5, we compare the effect of RanT24N and RanQ69L on microtubule polymerization around nuclei during NEBD in vitro. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200703002/DC1>.

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