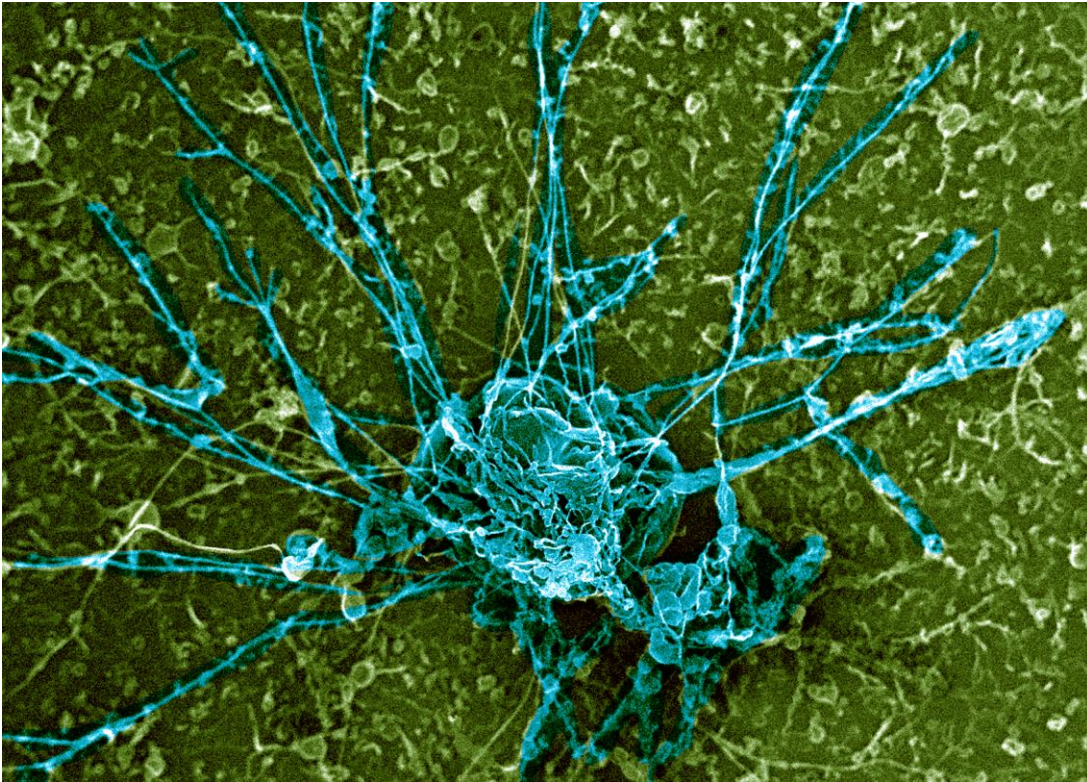


**Nuclear membrane disassembly and rupture.**

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### **Summary**

The nuclear envelope consists of two membranes traversed by nuclear pore complexes. The outer membrane is continuous with the endoplasmic reticulum. At mitosis nuclear pore complexes are dismantled and membranes disperse. The mechanism of dispersal is controversial: one view is that membranes feed into the endoplasmic reticulum, another is that they vesiculate. Using *Xenopus* egg extracts, nuclei have been assembled and then induced to breakdown by addition of metaphase extract. Field emission scanning electron microscopy, was used to study disassembly. Strikingly, endoplasmic reticulum-like membrane tubules form from the nuclear surface after the addition of metaphase extracts, but vesicles were also observed. Microtubule inhibitors slowed but did not prevent membrane removal, whereas Brefeldin A which inhibits vesicle formation, stops membrane disassembly, suggesting that vesiculation is necessary. Structures that looked like coated buds were observed and buds were labelled for  $\beta$ -COP. We show that nuclear pore complexes are dismantled and the pore closed prior to membrane rupturing, suggesting that rupturing is an active process rather than a result of enlargement of nuclear pores.

## Introduction

The nuclear envelope (NE) breaks down and reforms each cell cycle. The NE consists of the inner nuclear membrane (INM), the outer nuclear membrane (ONM) which are separated by the perinuclear space and punctuated by nuclear pore complexes (NPCs). The ONM shares proteins and properties with the endoplasmic reticulum (ER), whose lumen is continuous with it. The INM contains a distinct set of proteins, such as lamin B receptor (LBR) and LAP2 and the nuclear lamina (1). The lamina is a protein network thought to provide a structural framework for the NE and an anchoring site for interphase chromosomes but may also have a more active role in controlling nuclear organisation (2). Movement of macromolecules across the NE is regulated through the NPCs. NPCs are channels of ~120 MD and ~120 nm diameter in vertebrates (3) and may be smaller in yeast (4,5), but in many respects are conserved. The NPC has an eightfold rotational symmetry and comprises multiple copies of ~ 30 or more (6,7) nucleoporins. The structural framework of the NPC, consists of the spoke-ring complex, the central transporter (8), cytoplasmic and nucleoplasmic rings, attached cytoplasmic filaments and the nuclear basket, (9-14).

At mitosis, the NE membranes, NPCs and lamina are disassembled. They are then recycled to form new NEs around the chromatin in telophase and early G1. NE assembly has been characterised in a variety of model systems including *Xenopus* egg extracts. When sperm chromatin is added to interphase extracts it decondenses, membrane vesicles bind (16-18) in a cell-cycle regulated manner (18,19). Once bound, vesicles fuse to form a double membrane, NPCs assemble in flattened patches, and the membranes undergo “smoothing” which requires nuclear transport (17,20). NPC assembly is a stepwise process via intermediates such as dimples, pores, star-rings, thin rings and the cytoplasmic ring with cytoplasmic filaments (10, 21, 22). Older electron microscopy data suggested the formation of NPC-like structures (“pre-pores”) on the chromosome surface prior to membrane addition (23). More recently it was shown that NPC assembly required the attachment of the Nup107 complex to the chromatin surface prior to membrane assembly (24), which resurrected the pre-pore idea. In *Drosophila* embryos, NPC disassembly appears to be a stepwise reverse of the assembly process (22).

Several studies support the idea that NE-specific vesicles form during mitosis and remain separate from the ER (18, 25-29). However Ellenberg et al (1997) (30) demonstrated in living cells that during mitosis LBR-GFP was redistributed by diffusion from the NE into the ER, which remained intact. Yang et al (1997) (31) examined the fate of NE proteins in intact fixed mammalian cells and demonstrated that LAP1, LAP2 and gp210 were localized throughout the ER. They argued that temporal differences between gp210 and LBR binding to the NE during reassembly (25) may be due to the fact that their appropriate binding sites appear asynchronously and not due to the fact that the proteins are in distinct vesicles. In contrast, it has been shown that two distinct vesicle types, NEP-B and NEP-A, are necessary for NE assembly (18, 29). Recently it has become clear that the mitotic membranes used to assemble the NE during telophase/early G1 are distinct from bulk ER membranes because for their assembly they require the complex p97-UFD1-NPL4 (32) as well as Ran (33, 34, 35) whereas ER formation does not (33). However, this distinction could be achieved by creating microdomains in the ER (36).

Recently, it was found that NE breakdown may involve components of COPI, which are involved in forming vesicles during membrane trafficking. These components are recruited by nucleoporins (37,38). Therefore a role for vesicle formation in NE disassembly is distinctly possible. Coat proteins are recruited by the small GTPases ADP-ribosylation factors (ARFs) when in their active GTP-bound form. Brefeldin A inhibits nucleotide exchange factors that activate ARFs (39, 40) and inhibits NE breakdown (37) suggesting that ARFs have a role in NE disassembly.

Georgatos et al (1997) (41) showed that microtubules contribute to the disassembly of the NE but microtubule integrity is not necessary for nuclear lamina breakdown and membrane fragmentation (42). Microtubules appear to play a direct role in disassembly by forming nuclear indentations (41). Recent data showed that microtubules created tension on the NE which induced NE tearing and removal of the membranes towards the centrosomes (43) in a dynein dependent manner (44). Although microtubule driven ER dynamics are inhibited during metaphase (45) it may be possible that microtubules are utilised during prophase to direct the NE-linked ER away from the nucleus.

We have used feSEM to study the steps of NE disassembly in *Xenopus* egg extracts. We have found evidence for both the formation of ER-like tubules as well as vesicles during NE breakdown. Further evidence for the role of vesiculation is provided using drugs that disrupt microtubule and membrane dynamics. We also find that NPCs disassemble and the pore closes up prior to membrane rupturing. This challenges current models that propose that after removal of NPC proteins, the nuclear pores expand as the membranes are removed (46).

## **Results**

### **NE disassembly in *Xenopus* egg extracts**

To look at the structural changes of the NE during disassembly, nuclei were first assembled in interphase *Xenopus* egg extracts. Assembled nuclei are generally spheroidal, the DNA (stained with Hoechst 33258 – Fig. 1a left inset) appears diffuse throughout the nucleus and it is surrounded by a complete DHCC staining membrane (Fig. 1a right inset). The nuclear surface was examined using feSEM (Fig. 1a), and was somewhat convoluted and had many NPCs (Fig. 2a).

To induce metaphase an equal volume of metaphase extract was added to the assembly reaction. Chromatin condensation is the first most obvious change seen by light microscopy (insets in Fig.1b and d). The chromatin becomes a compact body in what appears to be a loose bag of membrane. This is demonstrated both by fluorescence microscopy (Fig. 1d inset) and by feSEM where the chromatin is seen as a dense elongated body “draped” in a loose membrane bag (delineated by dotted line in Fig. 1d).

### **ER tubules and vesicles emanate from the NE**

An early and striking event, seen most clearly by feSEM (Fig. 1b), is the appearance of ER-like membrane tubules, which in some, but not all, nuclei emanate mainly from one end of the condensing nucleus (Fig. 1c). In these nuclei the membrane tubules have an

“astral” appearance which led us to suspect a microtubule involvement in their organisation. Membrane tubules are approximately 200 nm in diameter (Fig. 2b and c) and are ER-like as they have ribosomes on their surface and are similar to the ER tubules that assemble in these extracts. Such ER tubules were previously observed by metal shadow TEM and labelled with antibodies to ER markers (45,47). One difference, however, is that the membrane tubules that are associated with the NE (Fig. 2b, large arrows) appear more flattened than general ER tubules which have a spherical profile. This suggests that proteins may be present in the NE membranes that are responsible for flattening. An NE tubule is compared to an ER tubule in Fig. 2e and f.

In addition to the ER-like tubules, many vesicles of varying size and ribosome density are at the nuclear surface (Fig. 2c and d). The vesicles appear to be budding from the NE. Some vesicles have many ribosomes on their surface whereas others have few or none.

It is possible that the vesicles could be remnants of membrane tubules damaged during specimen preparation. Therefore we looked for evidence of vesicles by light microscopy of “live” disassembling nuclei, which have not been fixed or isolated. Nuclei were assembled in egg extracts, a vital membrane fluorescent dye (BODIPY FL C<sub>5</sub>-ceramide) was added and the nuclei were induced to disassemble. We used confocal microscopy to look at optical slices of the disassembling nuclei (Fig. 2g). We detected numerous small membrane containing particles associated with the disassembling NE which could be vesicles. By scrolling through optical sections taken from the entire nucleus we could show that these were discrete particles and not sections through tubules. This suggests that the vesicles observed by feSEM are not artefacts of preparation.

At later stages, the membrane ruptures and much of it is removed revealing condensed chromatin (Fig. 2d). By the end of disassembly, some vesicles and tubules remain attached to the surface and the nucleus has become long, thin and sperm shaped (Fig. 2d). Rupture points can also be detected in optical sections (Fig. 2g).

### **Coated bud like structures are observed and a $\beta$ -COP antibody labels NE buds**

Recently it was shown that COPI complexes are involved in NE disassembly (37,38), but it was not shown whether this was for formation of coated vesicles from the nuclear membranes. We observed buds and vesicles attached to the disassembling NE (Fig. 2c and d). Intriguingly, we find structures that look like buds that are coated with particles (Fig. 2h and i) that are reminiscent of the published deep etch rotary shadow EM surface structure of COPII-coated vesicles (48). We suggest that these are indeed COPI-coated buds. To support this suggestion we immuno-gold labelled disassembling nuclei with an antibody to  $\beta$ -COP, using the same commercial antibody characterised by Liu et al. (2003) (37), and found a specific localisation of the gold particles on buds on the NE surface (Fig. 2j and k).  $\beta$ -COP labelling was observed at all times during membrane disassembly.

### **Disassembly of the NPCs**

The membrane surface of a fully assembled nucleus has many NPCs (Fig. 3a). At high magnification, the cytoplasmic filaments, cytoplasmic ring and some internal structures

of the NPC are visualised (Fig. 4a). The NPC has an external diameter of ~105 nm, consistent with that previously reported in feSEM studies of NPCs assembled in egg extracts (21). NPC disassembly is not synchronous and there are NPCs at different stages of disassembly present on the surface of a single nucleus (Fig.3 and Fig.4i).

Mature NPCs (Fig. 3a, Fig. 4a) begin to disassemble with the removal of the cytoplasmic filaments (Fig. 4a, stars) revealing the thin ring (Fig. 3b, TR, Fig. 4b). The star-ring (Fig. 3b, SR, Fig. 4c and d), which lies under the cytoplasmic ring (21,49), is infrequently seen during disassembly, suggesting it is rapidly removed. Stabilised pores are apparent (Fig. 3b and c, SP, Fig. 4e) and sometimes lose the central material (Fig. 4f). They become dimples (Fig. 3b and c, D, Fig. 4g and h) and finally close up to give an almost or completely pore-free membrane surface (Fig. 3c). This then ruptures, exposing the chromatin (Fig. 3d). The relative number of these different intermediates was quantified over time (Fig. 3f), showing the gradual shift from larger more complex structures to smaller simpler ones as the NE disassembles.

Although these various disassembling structures of the NPC are seen at fixed time points, we have placed them in order of decreasing complexity and size. This appears to be more-or-less a reversal of the assembly process previously reported in egg extracts (21) and is similar to disassembly in *Drosophila* early embryos (22). Also we often have observed “empty” central regions, particularly at the stabilised pore stage (Fig. 4f) which was not observed during assembly. This may be consistent with the first permeabilisation stage observed in starfish oocytes (50)

### **Rupturing of the NE appears to be independent from NPC disassembly**

Rupturing of the nuclear envelope generally occurs after completion of NPC disassembly and closing up of the pores (Fig. 2d and Fig. 3d). Generally, NPC disassembly was completed in 20-30 minutes after addition of metaphase extract. At this stage we observed nuclei with intact NE but with no NPCs or just a few late disassembly intermediates such as pores and dimples. 10-20 minutes later we found many nuclei with ruptured NEs, which were not observed at the earlier time period. Quantification of the total number of NPCs and intermediates at each time point (Fig. 3e) showed that NPC related structures decreased in number over time and hence pores were closing up. This suggested that NE rupture occurs after most nuclear pores have closed and argues against the possibility that rupture originates at the pores as previously suggested (46).

### **Inhibitors of membrane and microtubule dynamics affect NE breakdown**

The effects of several reagents that are expected to affect the processes of NPC and NE disassembly were examined by light microscopy and feSEM (Fig. 5). Nuclei were assembled in interphase extract then mixed with an equal volume of metaphase extract containing either Nocodazole, Brefeldin A, or GTP $\gamma$ S. Nuclei were sampled over time and prepared for light microscopy or feSEM.

### **Microtubule inhibitors slow down but do not inhibit nuclear membrane disassembly**

Nocodazole inhibits microtubule polymerisation. Microtubules have been shown to be involved in the formation of tubular ER networks in egg extracts (45) and have also been

shown to be involved in deformation of the nuclear membranes during mitosis (41, 43, 44). Therefore we wanted to test whether formation of membrane tubules observed during normal disassembly in egg extracts (Fig. 2) was dependent on microtubules and whether the formation of these membrane tubules was required for nuclear membrane disassembly.

Fluorescence microscopy showed that nocodazole (10  $\mu\text{g}/\text{ml}$ ) did not effect chromatin condensation, which occurred at the normal rate, but it did slow down membrane disassembly, and some membrane was often still present on the chromatin surface after several hours (inset Fig. 5d inset). After about one hour the membranes are still fully intact with Nocodazole (Fig. 5c inset) whereas they are difficult to detect by light microscopy in its absence.

Consistently, feSEM showed that the membrane is still intact at  $\sim 1$  hour of incubation in metaphase extract with Nocodazole (Fig. 5c) whereas during normal disassembly it would be mostly disassembled after 30 minutes (Fig. 3d). It then takes at least another 30 minutes to achieve the same level of disassembly in the presence of Nocodazole (Fig.5d). However, even after several hours there are some nuclei whose membrane has not ruptured, whereas during normal disassembly we only found nuclei that had ruptured. This suggests that rupturing was not always dependent on microtubules.

We do see membrane tubules in the presence of Nocodazole. In fact the steps of membrane disassembly appear very similar to normal disassembly, just slower. Therefore Nocodazole does not appear prevent the formation of membrane tubules. However it is possible that the reduction in membrane tubule movement under such conditions (51) could (partially) account for the slow down in membrane removal.

Nocodazole did not appear to significantly inhibit NPC disassembly.

### **Brefeldin A allows normal NPC disassembly but inhibits membrane disassembly**

Brefeldin A prevents the formation of the GTP bound form of ARF1 by inhibiting its nucleotide exchange factor (39, 40). This prevents the recruitment and assembly of coat proteins required for vesicle formation. However, it does not prevent the movement of membranes between for instance the Golgi and ER which instead form connections of tubular membranes. Therefore addition of Brefeldin A to a nuclear disassembly reaction should test whether vesicle budding is necessary for nuclear membrane disassembly.

Fluorescence microscopy shows that addition of Brefeldin A (5  $\mu\text{g}/\text{ml}$ ) to nuclear disassembly reactions caused a complete inhibition of chromatin condensation and membrane breakdown for several hours (Fig.5g inset). NPC disassembly appears to be normal as seen by feSEM (Fig. 5e and f). After 50-60 minutes where, in parallel controls, the membrane had been disrupted, and at least partially removed (Fig. 3d), the NPCs completely disassemble but the membrane was still fully intact (Fig. 5c), and remained so for several hours. Therefore in agreement with previous results (37,38) there is a Brefeldin A sensitive process that is required for the complete disassembly of the nuclear

membranes. The most likely candidate for such a process is the formation of coated vesicles. This suggestion is supported by our observation of  $\beta$ -COP containing membrane buds on the NE surface (Fig 2h-k). Brefeldin A does not appear to affect NPC disassembly in these experiments.

### **GTP $\gamma$ S slows membrane removal**

GTP $\gamma$ S, a non-hydrolyzable analogue of GTP is a potent inhibitor of nuclear envelope assembly and inhibits vesicle fusion and NPC assembly (20). Disassembly, however, involves tubule and/or vesicle formation (which we would not predict to be affected by GTP $\gamma$ S). Fluorescence microscopy (Fig. 5j inset) showed that although GTP $\gamma$ S had no effect on chromatin condensation, it slowed down membrane disassembly. At about one hour in normal disassembly the membrane is ruptured and mostly removed. However, in the presence of GTP $\gamma$ S most of the membrane still surrounds the nucleus, although some rupturing may occur (Fig.5j inset arrows). By later times, the membrane is completely removed in some nuclei (Fig. 5k) but not in others (Fig. 5l). Therefore, similar to Nocodazole, GTP $\gamma$ S slowed but did not completely inhibit membrane disassembly, suggesting a role for GTP hydrolysis in nuclear membrane disassembly.

## **Discussion**

### **Nuclear Membrane Disassembly**

The data presented here suggests that NE disassembly may be divided into the following events: (a) dispersal of nuclear membranes into the cytoplasm via vesicles; (b) dispersal of nuclear membranes into the ER via membrane tubules; (c) NPC disassembly; (d) sealing of the nuclear pore; (e) rupture of the NE.

### **NE is fed into the ER during disassembly**

ER-like tubules are formed at the NE surface during NE disassembly. This is consistent with previous studies demonstrating that NE integral membrane proteins move into the ER during mitosis (30, 31). It is uncertain if the tubules have grown out of the NE and hence are derived entirely from nuclear membranes, or if cytoplasmic ER tubules in the extract have been directed to fuse with the NE during prophase to allow the flow of nuclear membranes into the ER. These would be very different mechanisms. The first would be a process driven from the NE and might involve microtubules/cytoplasmic dynein (44) driven membrane tubule extension. The second would require extensive fusion of ER tubules with the NE, followed by passive flow of membranes released from the NE when protein structures such as the lamina and NPCs are disassembled.

Since inhibition of microtubule dynamics inhibits ER membrane tubule extension (52), we predicted that it would also inhibit nuclear membrane disassembly if this was dependent on tubule extension. We found that Nocodazole, a microtubule assembly inhibitor, failed to completely inhibit nuclear membrane disassembly (53). This indicated that nuclear membrane removal was not completely dependent on membrane tubule extension. However, the rate of NE breakdown was slowed, in agreement with other recent studies (43, 44), suggesting that microtubules are involved. It has been shown (41) that microtubule asters may contribute to the orderly disassembly of the NE by causing specific indentations in the NE in prophase. In the presence of Nocodazole, however, NE



disassembly appeared less ordered. Although evidence was not presented for any attachment of aster microtubules to any membranes, it is intriguing that we sometimes observed arrays of membranes that have the appearance of asters as if the NE/ER tubules are running along the astral microtubules. These findings suggest that microtubules aid but are not essential for NE disassembly and this may be due to a disorganisation of the membrane tubule streaming from the NE. Likewise, in the secretory pathway, microtubules are used, probably to increase the organisational efficiency, but are not essential (54). We did find that membrane tubules were still present during disassembly with Nocodazole, suggesting that tubule formation was not prevented when microtubules were inhibited. Therefore it seems likely that the role of microtubules may be to efficiently extend the membrane tubules away from the NE rather than in the initial formation of the tubules.

### **Vesicles also form at the NE during NEBD**

Different types of vesicles were also observed apparently budding from the NE. The different morphologies of these vesicles (rough and smooth) suggest that there may be a sorting process. Vigers and Lohka (1991) (55) identified two types of vesicles called NEP-A and NEP-B. It was proposed that NEP-B vesicles are targeted to the chromatin whereas NEP-A contribute to NE growth. In support of this, it was shown (29) that two functionally and biochemically distinct vesicle types are necessary for NE assembly.

We sort additional evidence for vesicle formation. We tested whether Brefeldin A, which inhibits vesicle formation in the secretory system, could also inhibit NE disassembly. Brefeldin A inhibits nucleotide exchange factors that activate ARFs (39, 40) which, in their active GTP-bound form, recruit and assemble the coat proteins essential for trafficking (reviewed in 56, 57). Brefeldin A inhibited membrane breakdown but not NPC disassembly, resulting in an intact and stable NE with no NPCs. The inhibition of NE disassembly by Brefeldin A suggests that disassembly may be regulated by a member of the ARF family. These results confirm previous findings (37) that Brefeldin A inhibits NE breakdown and that Arf may be involved (58). Liu et al. (2003) (37) confirmed the role of Arf with a dominant negative peptide of the protein and by immuno-depleting it. Conversely, Gant and Wilson (1997) (59) did not see an effect of Brefeldin A. We have found that the effects of Brefeldin A are concentration dependent and that the extracts are inherently variable, so these differences could be experimental variation. However, it is clear that in these experiments NE breakdown was affected by Brefeldin A suggesting a role for vesicle formation.

The recent finding that COPI complexes have a role in NE disassembly (37, 38) suggest that vesicle formation at the NE is driven by COPI coating. We observed what appeared to be buds on the surface of the outer nuclear membrane during NE disassembly. Sometimes these were smooth, sometimes they had ribosomes and occasionally a dense network presumably of protein was on the surface. It is possible that such a structure could be a COPI coat and indeed when we labelled disassembling NEs with an antibody to  $\beta$ -COP (a COPI component) we found that buds were often densely labeled with the antibody whereas the surrounding membrane was very lightly labeled if at all. This shows that  $\beta$ -COP is concentrated on the buds and suggests that at least some buds are formed

by COPI coating. It is possible that the buds that do not obviously have coats have already been fully formed and have been uncoated. We cannot rule out that alternatively there may be additional mechanisms or that there are some artefactual buds and vesicles. However it does seem clear that COPI driven vesicle formation is an important step in NE disassembly. Using feiSEM we can only observe events at the ONM surface. It is possible that  $\beta$ -COP also drives vesicle formation from the INM because it is recruited by the nucleoplasmic nucleoporin NUP153 (37,38), but this remains to be shown.

Interestingly, unlike the microtubule dependent step, the Brefeldin A sensitive step could not be by-passed. Although NPCs disassembled, the nuclei did not reduce in size and membranes appeared to remain intact. This could mean that tubule extension as well as vesicle formation is sensitive to Brefeldin A. This seems unlikely because in the secretory pathway Brefeldin A actually induces tubule formation (60). However, it could also be that the initial deformation of the flat outer nuclear membrane into tubules could be Brefeldin A sensitive. Alternatively, vesicle formation may have to start before extension of tubules can be initiated. This could be achieved via a biochemical checkpoint that only allows bulk flow of nuclear membranes into the ER after mitotic/meiotic vesicle formation is initiated. The purpose of such a checkpoint could be to prevent the bulk movement of nuclear membranes into the ER during interphase.

### **NPC Disassembly**

The NPC consists of several layers beginning on the cytoplasmic side with the cytoplasmic filaments, the cytoplasmic ring, the internal filaments and the star ring which lie above the central transporter and inner spoke ring complex (10, 11). On the nucleoplasmic side are internal filaments, nucleoplasmic ring and the basket. Disassembly of the NPC appears to occur via intermediates with each layer being removed roughly sequentially. This pattern of NPC disassembly mirrors, loosely, the model of NPC assembly proposed previously (21) where the NPC was proposed to assemble in a modular and stepwise fashion. For disassembly, the reverse appears to happen with the gradual removal of the cytoplasmic ring subunits followed by the thin ring subunits etc. leaving a structure of decreasing complexity until the NPC is reduced to a pore which then disappears. This confirms observations we made previously in *Drosophila* early embryos (22).

### **Rupturing the NE**

NPCs and intermediates were often not observed on ruptured NEs, suggesting that nuclear pores had closed up before rupturing. We also observed NPC-free nuclei prior to rupturing, whereas at later time points all nuclei had ruptured. This suggests that nuclei with no NPCs had ruptured and that the rupturing mechanism was distinct from NPC disassembly and did not involve expansion of nuclear pores. We find that generally NPCs are removed and the pore is closed up before the NE is ruptured. This is not consistent with models that propose that rupturing is facilitated by the removal of the NPC, leaving the pore which then expands as membrane is removed (46). Indeed we never see expanded pores. It has been shown by time-resolved confocal microscopy that the NE is permeabilised during NE breakdown in a two step process (52). 70kD fluorescent Dextran was injected into a starfish oocyte prior to NE breakdown. Before breakdown

starts, the Dextran cannot diffuse through the NPC (it is too large). It then initially enters slowly in the first phase followed by a rapid entry phase. It was argued that the first slow phase is a result of the partial dismantling of the NPC to give a small but free diffusion channel, which would be consistent with the “empty” stabilised pores that we observe. The second rapid phase was suggested to be enlargement of the pores. We suggest that the second phase is due to a rupturing event, possibly from a single point which results in a rapidly expanding hole, independent of NPC disassembly. As well as our data, the fact that rupturing occurs at only one or two points (52) rather than a multitude of points that would result from an enlargement of many nuclear pores supports this view. Although it makes intuitive sense that the membrane removal should be facilitated by pore expansion, there is no direct evidence to our knowledge that supports this idea. Moreover such a mechanism would still result in a network of tubules surrounding the chromosomes which would have to be fragmented by multiple fission events. We never observe such a network. Fig. 6 shows the sequence of events that we suggest occur during NE breakdown.

## **Materials and Methods**

### **Buffers and reagents**

Lysis buffer: 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 10 mM Hepes (pH 7.58), 50 µg/ml cycloheximide, 5 µg/ml cytochalasin B, 10 µg/ml aprotinin, 10 µg/ml leupeptin. Membrane wash buffer (MWB): 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 50 mM Hepes - NaOH (pH 7.4), 0.5 M sucrose, 12 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin. M lysis buffer: 240 mM β-glycerophosphate (pH 7.4), 60 mM EGTA (pH 8), 45 mM MgCl<sub>2</sub>, 1 mM DTT. Ringers solution: 100 ml ringers A (111 mM NaCl, 1.9 mM KCl, 1.1 mM CaCl<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>), Hepes 5:1 buffer: 83 mM KCl, 17 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 mM Hepes, pH 7.4. Membrane fix: 80 mM Pipes - KOH (pH 6.8), 1 mM MgCl<sub>2</sub>, 150 mM sucrose, 2% paraformaldehyde (v/v), 0.25% glutaraldehyde (v/v). Tannic acid fix: 2% glutaraldehyde (v/v), 0.2% tannic acid (w/v) in 100 mM Hepes, pH 7.4. Extract buffer (EB): 100 mM KCL, 5 mM MgCl<sub>2</sub>, 20 mM Hepes, pH 7.5, 2 mM 2-mercaptoethanol.

### **Preparation of egg extracts from *Xenopus***

Cell-free extracts were prepared from *Xenopus* activated (interphase extracts) or unactivated (metaphase extracts) eggs as previously described (61, 62). Briefly, eggs were lysed by centrifugation at 10,000 g for 12 min, washed in 0.1 M NaCl and rinsed in either lysis (interphase extracts) or M lysis buffer (metaphase extracts). Cytoplasm was spun at 30,000g for 10 min and supplemented with an ATP regenerating system (1 mM ATP, 50 µg/ml creatine phosphokinase, 10 mM creatine phosphate). Aliquots were frozen in liquid nitrogen and stored at -80°C.

### **Nuclear assembly and disassembly reactions**

Assembly reactions were initiated by addition of sperm chromatin, at 2,000 sperm/µl extract. Assembly reactions were incubated at room temperature (22-24°C) for up to 3 hours before initiation of the nuclear disassembly. Disassembly of assembled nuclei was initiated by addition of an equal volume of metaphase extract. The final concentration of

nocodazole in the disassembly reaction was 10 µg/ml. Taxol was added to a final concentration of 10 µM. GTPγS was added to a final concentration of 2 mM and GTP at the same concentration was a control. Brefeldin A was added to a final concentration of 5 µg/ml. N-ethyl maleimide (NEM), was added to a final concentration of 5mM. Excess NEM was quenched by the addition of 2 µl of 125 mM DTT. As a control DTT and NEM were mixed together 2 min before addition to the assembled extract.

### **Preparation of nuclei for feSEM**

Samples were prepared for analysis by feSEM as previously described (21). Briefly, aliquots (4µl) were resuspended in MWB and spun onto silicon chips at 3,000 rpm for 10 min at 4°C. Specimens were then fixed in Membrane fix for 10 min, washed in 0.2 M cacodylate pH 7.2, fixed in 1% OsO<sub>4</sub> in 0.2 M cacodylate for 10 min. After rinsing twice in water, samples were finally stained in 1% uranyl acetate for 10 min. Specimens were then dehydrated in a series of increasing alcohol concentrations (30-100%) followed by immersion in Arklone and critically point dried. Finally, specimens were sputter-coated with a 3nm of chromium or tantalum and then examined in a Topcon (ABT) ISI-DS-130F field emission scanning electron microscope (Topcon Corporation, Tokyo, Japan) at 30 kV accelerating voltage. Some samples were coated with a Cressington 308 chromium coater and examined with a Hitachi S5200 feiSEM.

### **Immuno-gold feSEM**

Nuclei were spun onto chips as above and fixed in membrane fix with the gluteraldehyde omitted for 10 minutes. Chips were washed in PBS and placed in 100mM glycine in PBS for 10 minutes, washed again and transferred to PBS with 1% fish skin gelatine (Sigma) for 1 hour. They were then incubated with the COPI antibody (Affinity Bio Reagents) at a 1:20 dilution in PBS for 1 hour, washed 4 times for 5 minutes in PBS then incubated with 5nm gold secondary (Amersham) for 1 hour and washed 4 times 5 minutes in PBS. Samples were then fixed again in membrane fix and processed for feSEM as above. To determine the position of the gold particles, a backscatter electron image was obtained at the same time as the secondary electron image. Gold particles appear as bright white dots in the backscatter image. Using Adobe Photoshop, the backscatter image was pasted as a separate layer onto the secondary image. This allows us to accurately mark the position of the gold particles on the secondary image, before removing the backscatter image. The positions of the gold particles are shown as yellow dots.

### **Fluorescence microscopy**

1µl extract was placed on a slide and mixed with an equal volume of MWB containing 10µg/ml Hoechst 33258 and 10µg/ml 3,3-dihexyloxycarbocyanine iodide (DHCC), then viewed on a fluorescent microscope.

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### Figure legends

Figure 1. Nuclear disassembly in *Xenopus* egg extracts. (a) FeSEM image of nucleus assembled in interphase extract. Left inset is fluorescent DNA stain (Hoechst 33258) of an interphase nucleus and right inset is DHCC membrane stain of the same nucleus. (b) Early disassembly showing extensive formation of membrane tubules (tail-less arrows) and vesicle buds (tailed arrows). Fluorescence microscopy also shows DHCC (green) stained membrane tubules attached to the nucleus (inset) (c) Sometimes membrane tubules emanate predominantly from one end with an "astral" appearance. (d) chromatin (CC) condenses rapidly leaving a loose bag of membrane draped over it (delineated by dotted line), which can also be seen by fluorescence microscopy (insets: right is DNA,

middle is membrane and left is combined image). (e) membrane is then removed and eventually ruptured (right inset). (f) after rupturing, the membrane fragments and is mostly removed (right inset) and the chromatin is exposed. Bar in (e) = 1.67 $\mu$ m refers to all images.

Figure 2. FeSEM of disassembly at high magnification. (a) Interphase nucleus, small arrows indicate NPCs. (b and c) Early disassembly, large tail-less arrows are ER-like tubules, large tailed arrows are ribosome-free vesicles, small tailed arrows are ribosome containing vesicles. (d) Ruptured NE showing exposed chromatin (Chr). (e) Flattened membrane tubule emanating from nucleus compared to (f) ER tubule in cytosol. (g) Confocal optical section of NE after rupture (arrowhead) showing putative vesicles (arrows). (h and i) Buds with coat-like structures. (j and k) Buds labelled with anti- $\beta$ -COP immunogold label (yellow dots).

Figure 3. Disassembly of NPCs over time. There is a gradual shift from mature NPCs (a) to dimples (b) to NPC-free membrane (c) which subsequently ruptures (d). TR, thin ring; SR, star ring; SP, stabilised pore; D, dimple. (e) Quantification of the number of NPCs per  $\mu$ m<sup>2</sup> during disassembly (total number counted t=0 minutes, 302; t=10 minutes, 137; t=20 minutes, 37. (f) change in relative percentage of different types of intermediate over time.

Figure 4. Proposed intermediates involved in NPC disassembly. (a) Interphase NPC showing cytoplasmic filaments (stars) and central structures. (b) Thin ring is visible after removal of cytoplasmic ring subunits. (c) star ring exposed. (d) star ring partially dismantled. (e) Stabilised pore. (f) central structures completely removed, edge of hole has rigid appearance which is probably part of the inner spoke ring (tailed arrow). (g) Edge of the hole becomes smooth presumably as the inner spoke ring is dismantled (arrow). (h) hole shrinks and presumably closes.

Figure 5. (a-d) Disassembly in the presence of Nocodazole. (a) early (15 minutes after adding mitotic extract), (b) mid (30 minutes), (c) late (60 minutes) and (d) 90 minutes. (e-g) same time points as (a-c) but in the presence of Brefeldin A. (h-l) disassembly with GTP $\gamma$ S over same time period as (a-d). The insets show equivalent nuclei in the light microscope stained for DNA (left) or membrane (right).

Figure 6. Model for NE disassembly. (a) Lamina disassembles, coated buds form, NPCs start to disassemble. (b) vesicles pinch off, NPCs disassemble and ER tubules extend from the NE along microtubules (rods). (c) Membrane tubules continue to extend and vesicles continue to bud off as the nuclear pores close up. (d-f) membrane ruptures exposing the chromatin.

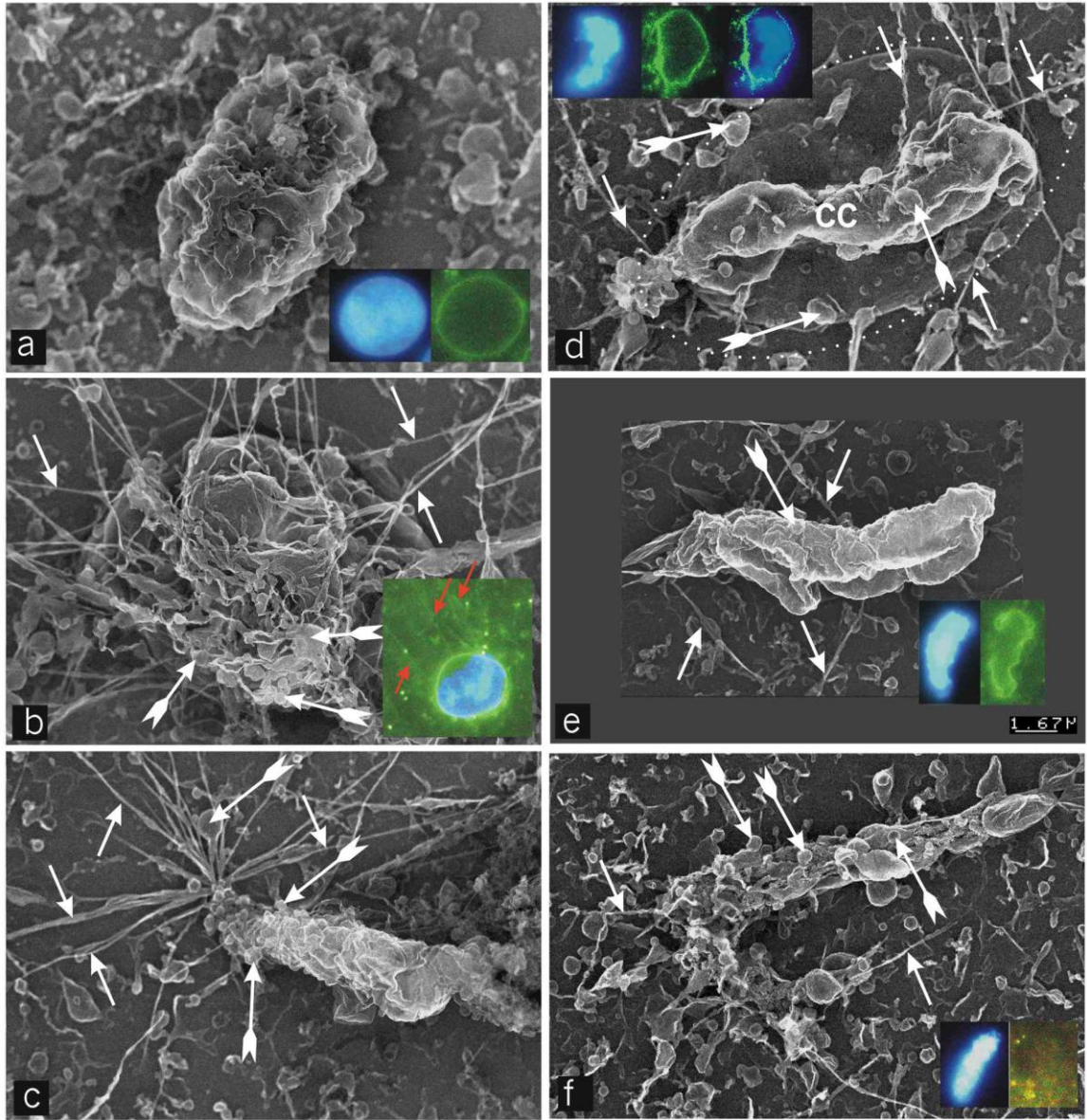


Figure 1

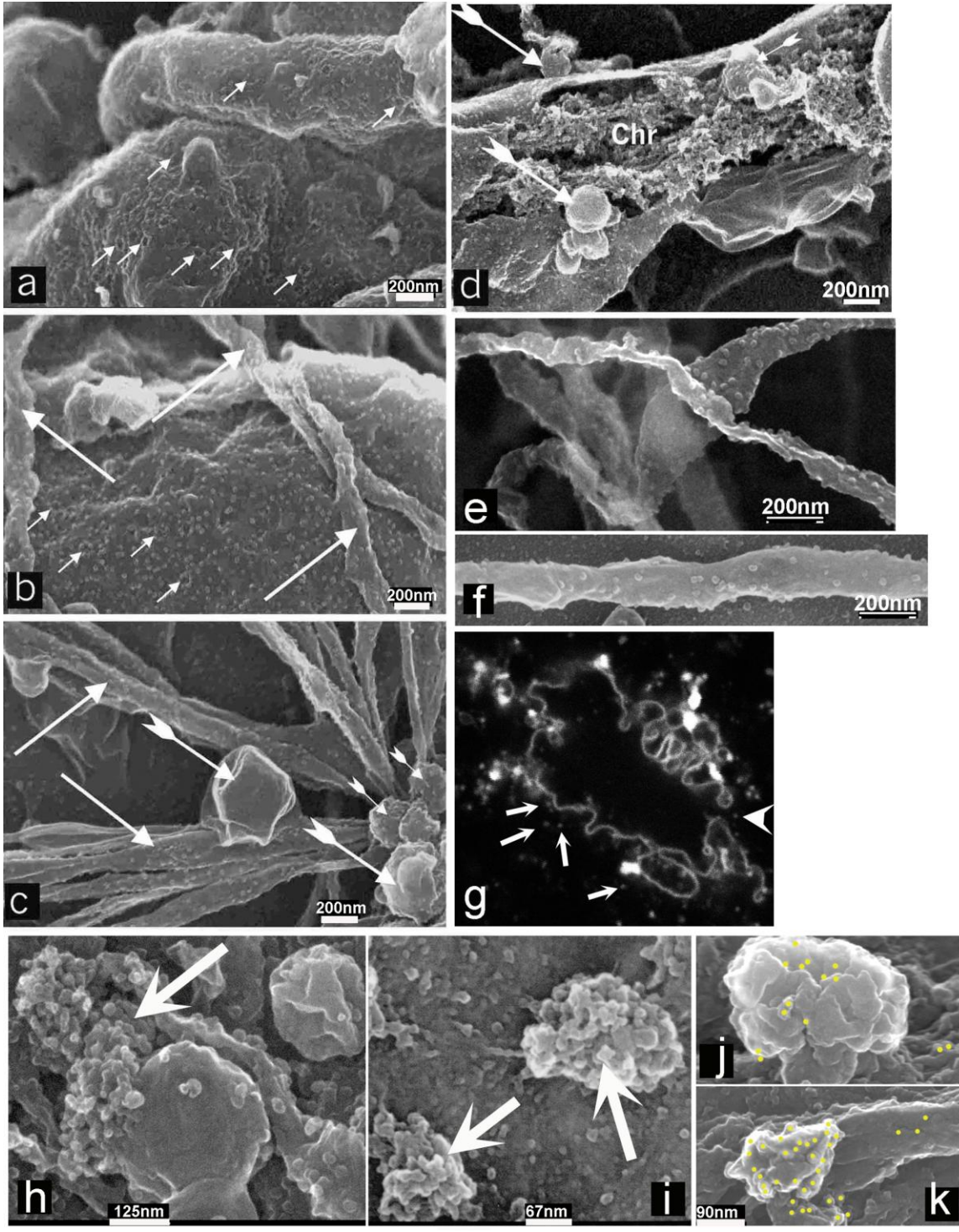


Figure 2

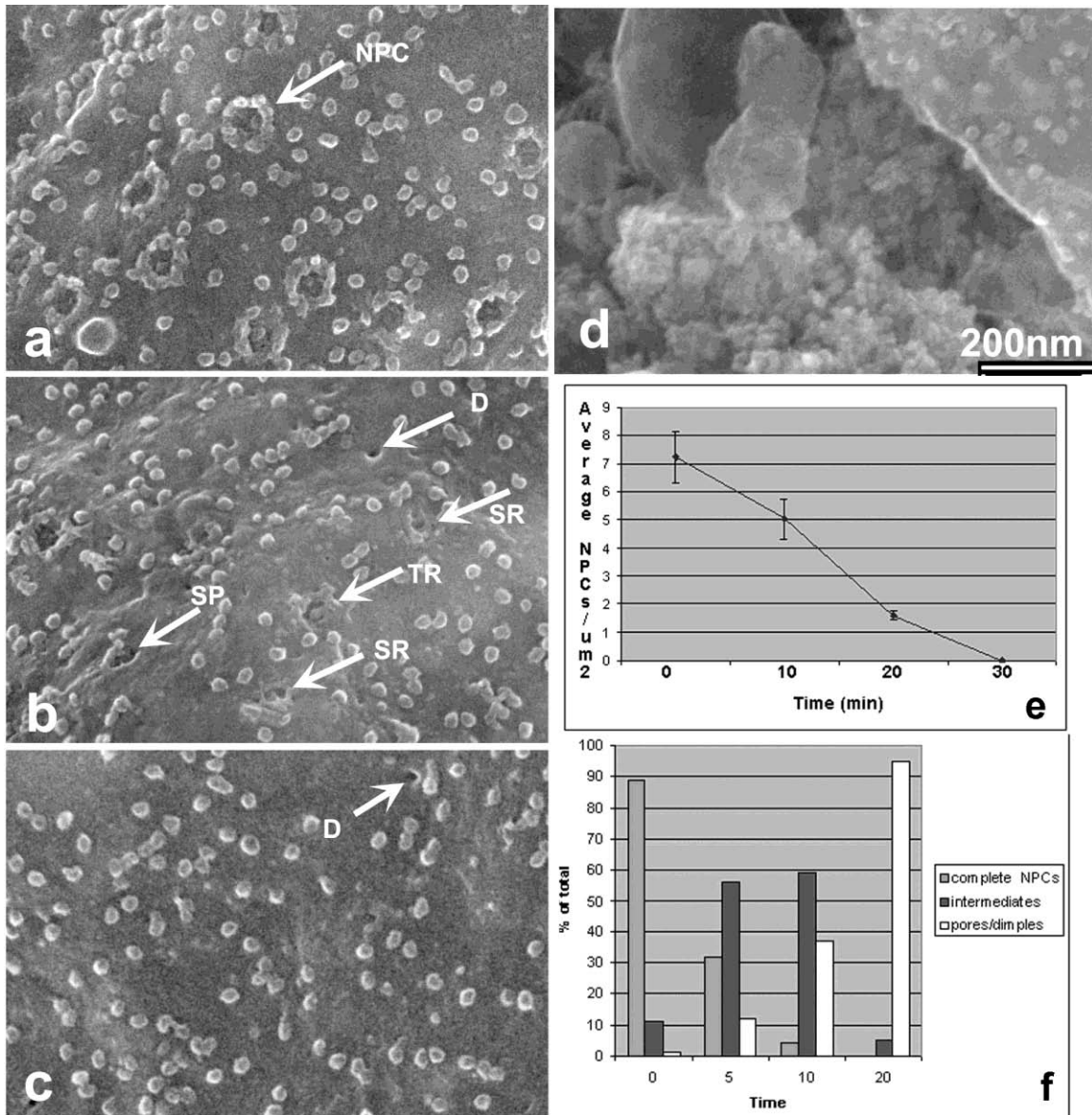


Figure 3

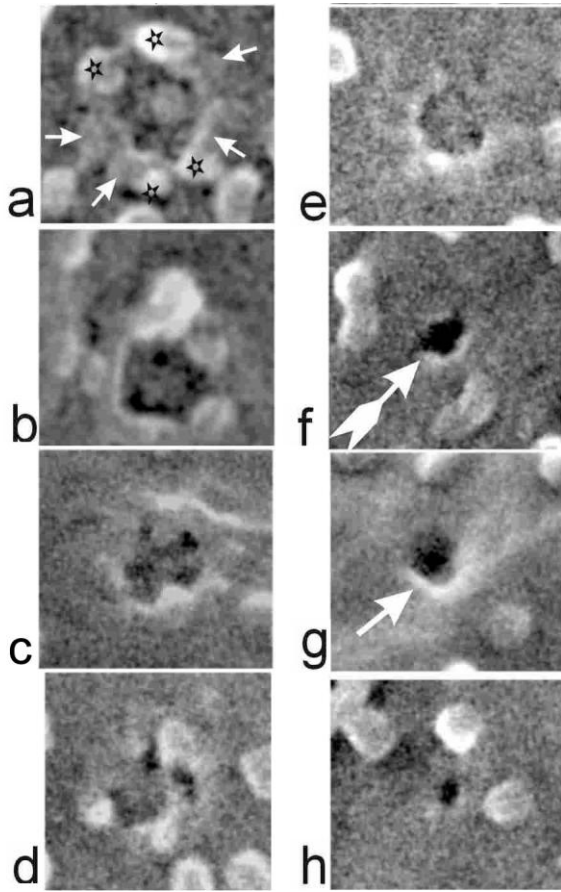


Figure 4

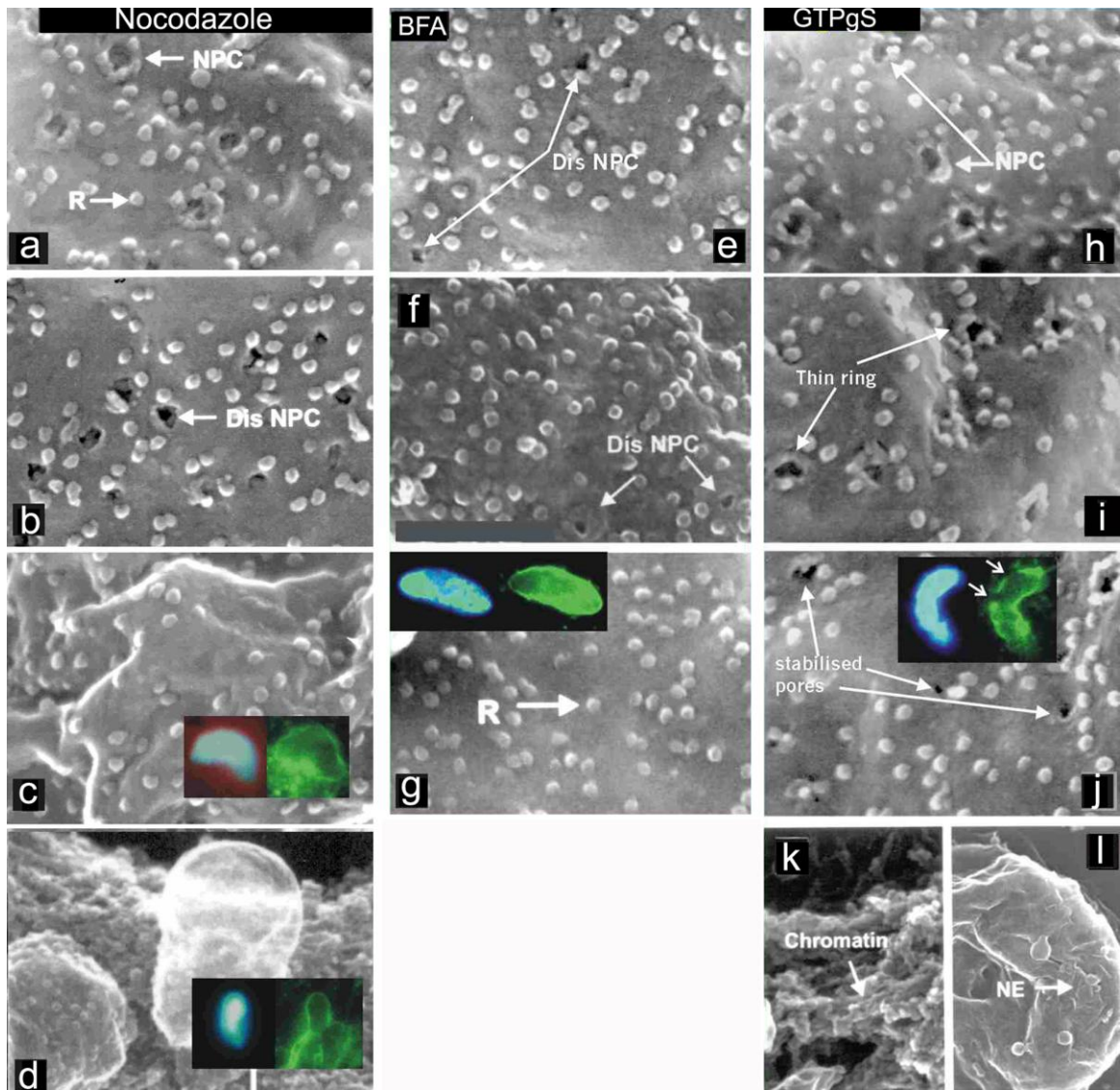


Figure 5

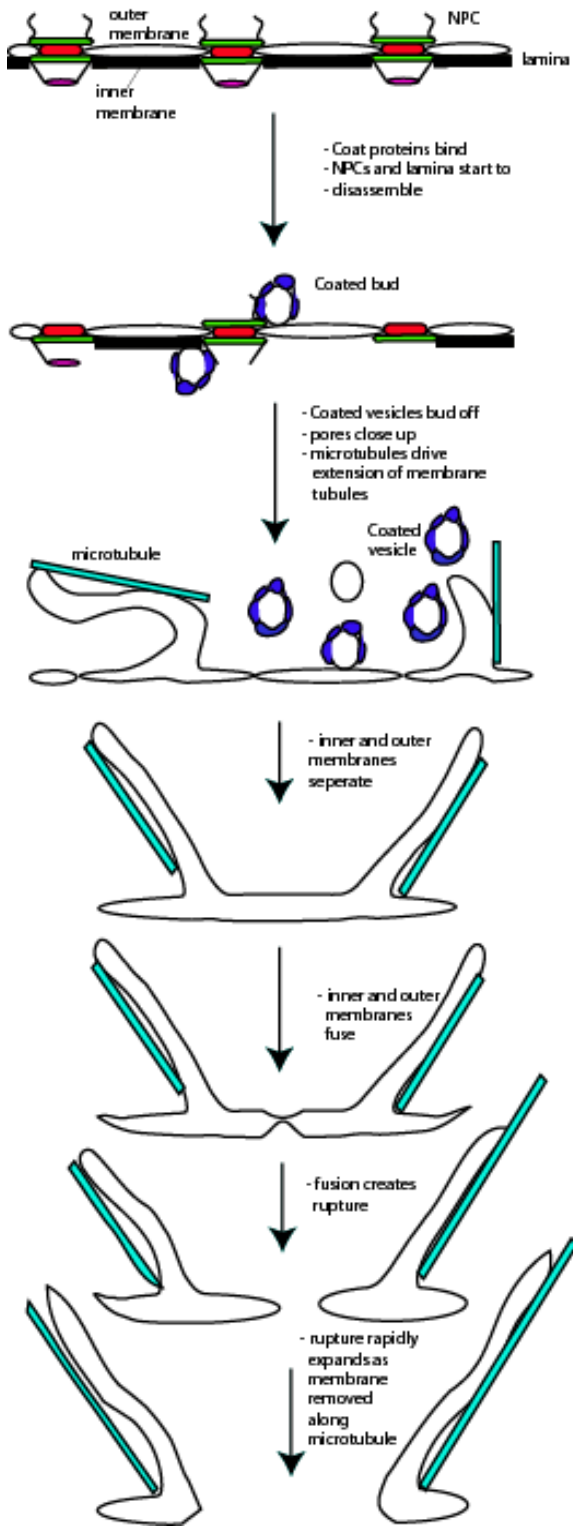


Figure 6