Steps of nuclear pore complex disassembly and reassembly during mitosis in early *Drosophila* embryos

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SUMMARY

The mechanisms of nuclear pore complex (NPC) assembly and disassembly during mitosis in vivo are not well defined. To address this and to identify the steps of the NPC disassembly and assembly, we investigated *Drosophila* embryo nuclear structure at the syncytial stage of early development using field emission scanning electron microscopy (FESEM), a high resolution surface imaging technique, and transmission electron microscopy. Nuclear division in syncytial embryos is characterized by semi-closed mitosis, during which the nuclear membranes are ruptured only at the polar regions and are arranged into an inner double membrane surrounded by an additional 'spindle envelope'. FESEM analysis of the steps of this process as viewed on the surface of the dividing nucleus confirm our previous in vitro model for the assembly of the NPCs via a series of structural intermediates, showing for the first time a temporal progression from one intermediate to the next. Nascent NPCs initially appear to form at the site of fusion between the mitotic nuclear envelope and the overlying spindle membrane. A model for NPC disassembly is offered that starts with the release of the central transporter and the removal of the cytoplasmic ring subunits before the star ring.

Key words: Nuclear pore complex, Nuclear envelope, *Drosophila*, Mitosis, Assembly, Disassembly, Embryo

INTRODUCTION

One of the most dramatic events of mitosis is the disassembly of the nuclear envelope (NE), which involves dismantling the nuclear pore complexes (NPCs) and the lamina and removal of the nuclear membranes, followed by its rapid reassembly as the cell leaves mitosis. This is a complex, orchestrated, fundamental process, and we understand little of its mechanism. Three different types of mitosis are known at present. Open mitosis involves breakdown of the nuclear membranes and disassembly of the NPCs and lamina in prophase, and their reassembly around the daughter chromosomes in telophase (Roos, 1973; Zeligs and Wollman, 1979; Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Meier and Georgatos, 1994; Wiese et al., 1997). Lower organisms, such as yeast, undergo 'closed mitosis', in which the NE appears to remain intact throughout (Byers, 1981) and goes through a division process. The nuclei of syncytial early *Drosophila* embryos go through a semi-closed mitosis, in which the NPCs disassemble but the chromosomes remain enclosed within the nuclear membranes, which are only disrupted at the poles (Stafstrom and Staehelin, 1984).

The NPC (Goldberg and Allen, 1995; Goldberg et al., 1999, Kiseleva et al., 2000; Allen et al., 2000; Stoffler et al., 1999) has a diameter of ~120 nm and is composed of a series of concentric rings with apparent eightfold symmetry in top view and double symmetry in side view (Unwin and Milligan, 1982; Akey and Radermacher, 1993). Within the pore is the inner spoke ring, which has a central aperture of ~40 nm. Within this aperture is the central transporter, which was found to alter conformation during transport (Akey, 1990; Kiseleva et al., 1998). The existence of the transporter is, however, still controversial and could represent in transit material. Eight spokes radiate out from the inner spoke ring, penetrating the pore membrane, and are joined together in the lumen by the radial arms, which might contain the nucleoporin gp210. On the cytoplasmic side of the NPC is the star ring, which is embedded in the membrane and underlies the cytoplasmic ring. The cytoplasmic ring consists of a thin ring with eight subunits moulded onto it and short rod-shaped particles extending into the cytoplasm, which are involved in import (Panté and Aebi, 1996; Rutherford et al., 1997) and contain the nucleoporin Nup358, which binds the nuclear transport factor Ran. A basket-like structure (Ris, 1997; Goldberg and Allen, 1996) is attached to the nucleoplasmic face, which forms an additional basket ring during mRNP export (Kiseleva et al., 1996) and contains the nucleoporin Nup153. Internal filaments join both the cytoplasmic and the nucleoplasmic rings to each end of the central transporter (Goldberg and Allen, 1996; Kiseleva et al., 1998).

The NPC consists of between 30 (Rout et al., 2000) and 100 (Reichelt et al., 1990) different nucleoporins, some of which are solubilized during mitosis by hyperphosphorylation (Favreau et al., 1996; Macaulay et al., 1995). Recruitment of these proteins during post-mitotic reassembly is sequential (Bodoor et al., 1999). One integral membrane nucleoporin,

POM121, is recruited early, whereas gp210 is a late arrival. The putative basket nucleoporin Nup153 is recruited to the chromatin early in anaphase, with Nup62 and Nup214 being added sequentially later in telophase (Bodoor et al., 1999), although Smythe et al. (Smythe et al., 2000) showed that Nup153 was incorporate later than Nup62 and Nup214 in *Xenopus* egg extracts, and was dependent on lamina assembly. It has also been shown in HeLa cells that various nucleoporins and nuclear membranes accumulate at the nuclear periphery in early telophase, a few minutes before the restoration of nuclear import function (Haraguchi et al., 2000). Integral proteins of the inner nuclear membrane become dispersed into the endoplasmic reticulum (ER) during mitosis (Ellenberg et al., 1997; Yang et al., 1997), suggesting that nuclear membrane disassembly occurs by feeding it into the ER network, although there is also evidence for vesiculation and sorting of these membranes (Warren, 1993; Collas and Courvalin, 2000; Drummond et al., 1999).

The process of NE assembly and disassembly has been studied using extracts from *Xenopus* eggs (Lohka, 1988; Wiese and Wilson, 1993). In this system, assembly starts with binding of at least two classes of vesicles to a chromatin surface (Vigers and Lohka, 1991; Drummond et al., 1999), which then fuse and flatten (Wiese et al., 1997). NPCs then assemble and we have previously identified putative assembly intermediates in this process (Goldberg et al., 1997a, Gant et al., 1998) using field emission scanning electron microscopy (FESEM). In this model, NPC assembly is initiated by invagination of the inner and outer membranes until they meet and fuse to create a pore. The pore is then stabilized, possibly by the assembly of parts of the spoke ring complex. Central material (probably the transporter) is inserted simultaneously with a build up of the components of the rings, and is followed by addition of the peripheral filaments. Although NPC assembly appears to require flattening of the membrane onto the chromatin, it does not (in *Xenopus* egg extracts) require the lamina (Goldberg et al., 1995; Newport et al., 1990) and can be inhibited by agents such as GTPγS, BAPTA (which chelates Ca^{2+} and Zn^{2+}) and wheat-germ agglutinin (which binds some nucleoporins) (Macaulay and Forbes, 1996; Goldberg et al., 1997a; Wiese et al., 1997; Finlay and Forbes, 1990; Dabauvalle et al., 1990).

In order to test our model based on in vitro data, we hoped to find evidence for a temporal progression from one proposed intermediate to the next and to check whether these same intermediates could be found in an in vivo system. Early embryos of *Drosophila melanogaster* provide a useful tool for answering these questions. Pre-blastoderm embryos go through 13 almost synchronous nuclear divisions, resulting in 6000 syncytial nuclei before cellularization (Rabinowitz, 1941). NPCs disassemble in prophase, are dispersed into the cytoplasm (Harel et al., 1989) and reassemble in telophase and G1. The nuclear membranes, however, remain intact, except for being ruptured at the poles to allow access of the spindle microtubules to the chromosomes (Stafstrom and Staehelin, 1984). The ruptured part of the envelope appears to form into vesicles, which contain the inner membrane proteins otefin and lamins, and form a second envelope (the 'spindle envelope') around the dividing nucleus throughout mitosis (Padan, 1990; Paddy et al., 1996).

Here, we have isolated nuclei at different stages of the cell cycle and mitosis from embryos and used FESEM and TEM to examine NE and NPC structure through mitosis of stages 11-13 of embryo development. We have been able to confirm the existence of the intermediates in vivo and for the first time provide evidence for the temporal progression during assembly from simpler smaller structures such as dimples and holes to larger more complex structures (mature NPCs), via intermediate structures like the star ring. Disassembly appears to be more rapid and synchronous and could be roughly a reverse of the assembly process, although it remains to be shown how closely it mirrors it. We also found that nascent NPCs assemble in clusters, apparently at the sites of fusion between the mitotic nuclear envelope and the spindle envelope.

MATERIALS AND METHODS

Handling embryos and analysis of stage of development

Eggs from two strains of wild-type *Drosophila melanogaster* females (Canton-S and Oregon R) were collected on trays inserted into culture flasks, covered with a layer of fresh yeast and kept at 25°C. Most eggs were collected at intervals from 1 to 2 hours after fertilization. Stages of development were selected by examination of their morphology under an inverted light microscope (Rabinowitz, 1941) or from the number of nuclei or spindles in cross sections of plastic embedded embryos. Additionally, fluorescent staining of DNA in embryos with Hoechst 33258 (1 μg ml^{-1} in PBS buffer; 4 minutes incubation at room temperature) was used. The nuclei from 25 different embryos were investigated by FESEM and TEM for several stages of mitosis, including: late interphase, prophase, early telophase, late telophase and early interphase (five embryos per stage). Nuclei at metaphase and anaphase were investigated only by TEM because their NE was discontinuous and such nuclei became disrupted during their isolation.

Sample preparation for TEM analysis

A method modified slightly from those previously described (Stafstrom and Staehelin, 1984) was used to fix embryos for TEM sections. Embryos were dechorionated manually on sticky tape, and floated for a short time on cold distilled water. A solution containing 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) was shaken with an equal volume of heptane and embryos were fixed in the heptane phase for 5 minutes. They were then transferred to an aqueous fixative containing 2.5% glutaraldehyde for 2 hours, during which time their vitelline membranes were removed with fine tungsten needles. Sodium cacodylate (0.05 M) was used for this and subsequent steps. Washing in buffer (three washes of 10 minutes each) was followed by post-fixation in buffered 1% OsO_4 for 1 hour. Embryos were then washed with buffer and stained in 1% aqueous uranyl acetate overnight at 4°C. The samples were then dehydrated and embedded in epoxy resin. Tangential sections of embryos were stained with uranyl acetate and lead citrate and analysed in a JEOL-1212 (Japan) transmission electron microscope.

FESEM

Dechorionated embryos were transferred onto silicon chips coated with poly-L-lysine in 10 μl of 10 mM Tris-HCl pH 7.2, 2.5 mM $MgCl_2$. The contents of the embryos were released with a dissecting needle and, within a few seconds, fixed for 10 minutes in 2.5% glutaraldehyde, 10 mM Tris-HCl, 2.5 mM MgCl, pH 7.2. The samples were centrifuged at 800 ***g*** to provide good attachment of nuclei to the chip surface and fixed with the same primary fixative and post-fixed in 1% OsO_4 buffered in 10 mM Tris-HCl. Further processing and FESEM analysis were as described by Goldberg and Allen (Goldberg and Allen, 1992).

Statistical analysis

The relative proportions of each putative NPC intermediate were calculated for each stage of mitosis from FESEM images. The data presented were derived from nine individual Canton S strain embryos. Each sample, prepared from a single embryo, contained nuclei at varying stages of mitosis (owing to the mitotic wave), which were morphologically very distinct and enabled us to define the stage of mitosis that they were at (see beginning of Results section and Figs 1,2,4,5,7, summarized in Fig. 8). Basically, early interphase nuclei were ~5 μm in diameter and monolobal. In early prophase, they were ~9 μm in diameter and monolobal. As separation began in early telophase, they became bilobal with two 4-5 μm lobes. These lobes became further separated in late telophase. Areas for analysis were selected randomly at low magnification, at which the NPCs were too small to see (to reduce bias in selection of the area). Images were then acquired at 60,000× magnification, and we defined a consistent sized area that was applied to each micrograph. We then counted the number of each intermediate in the selected area and converted this to a percentage. The data presented represent five datasets (micrographs) for each time point (stage of mitosis). We then calculated the mean percentage of each intermediate and the standard deviation over the five datasets. A total of 329 structures were counted.

RESULTS

In a previous study, we showed that, during in vitro nuclear assembly in *Xenopus* egg extracts, there were structures that could be intermediates in the assembly of the NPC (Goldberg et al., 1997a). To test this model, we wanted to see whether these same intermediates could be found in vivo and whether we could find evidence of a progression from one intermediate to the next. We chose to study early *Drosophila* embryos because it is an in vivo system in which nuclei divide and develop (almost) synchronously, and from which intact nuclei can be isolated for FESEM analysis.

Nuclear size and morphology in FESEM and TEM allow us to estimate which stage of the cell cycle nuclei are at, as follows. (1) Mid-interphase (Fig. 1): nuclei are roughly spherical, ~5 μm diameter and have many mature NPCs. (2) Prophase (Fig. 2): nuclear size increases to ~9 μm, there is a high density of partially dismantled NPCs and, as prophase proceeds, the polar regions of the nucleus start to vesiculate as the nucleus enters metaphase (Fig. 3). (3) Early telophase (Fig. 4): nuclei consist of two 4 μm diameter lobes and NPCs are incomplete and of lower density. (4) Late telophase (Fig. 5): daughter nuclei have almost separated and NPCs have increased in number and maturity. (5) Early interphase (Fig. 7): ~4 μm diameter nuclei have many mature and incomplete NPCs. We were unable to examine metaphase nuclei by FESEM as they were too fragile to be isolated, but thin sections of metaphase cells could by studied by TEM (Fig. 3). The process is summarized in Fig. 8.

Although we are building dynamic models on static FESEM images, we believe that this is a justifiable approach because the morphology of the nuclei are so distinct and can be correlated to well-established light microscopy (Baker et al., 1993) and TEM (Stafstrom and Staehelin, 1984) images of the stages of mitosis. Isolation, fixation and other procedures used for sample preparation probably have some effect on nuclear envelope and NPC structure but we believe that the changes observed do reflect the real dynamics of NE morphology during mitosis for each stage relative to the others.

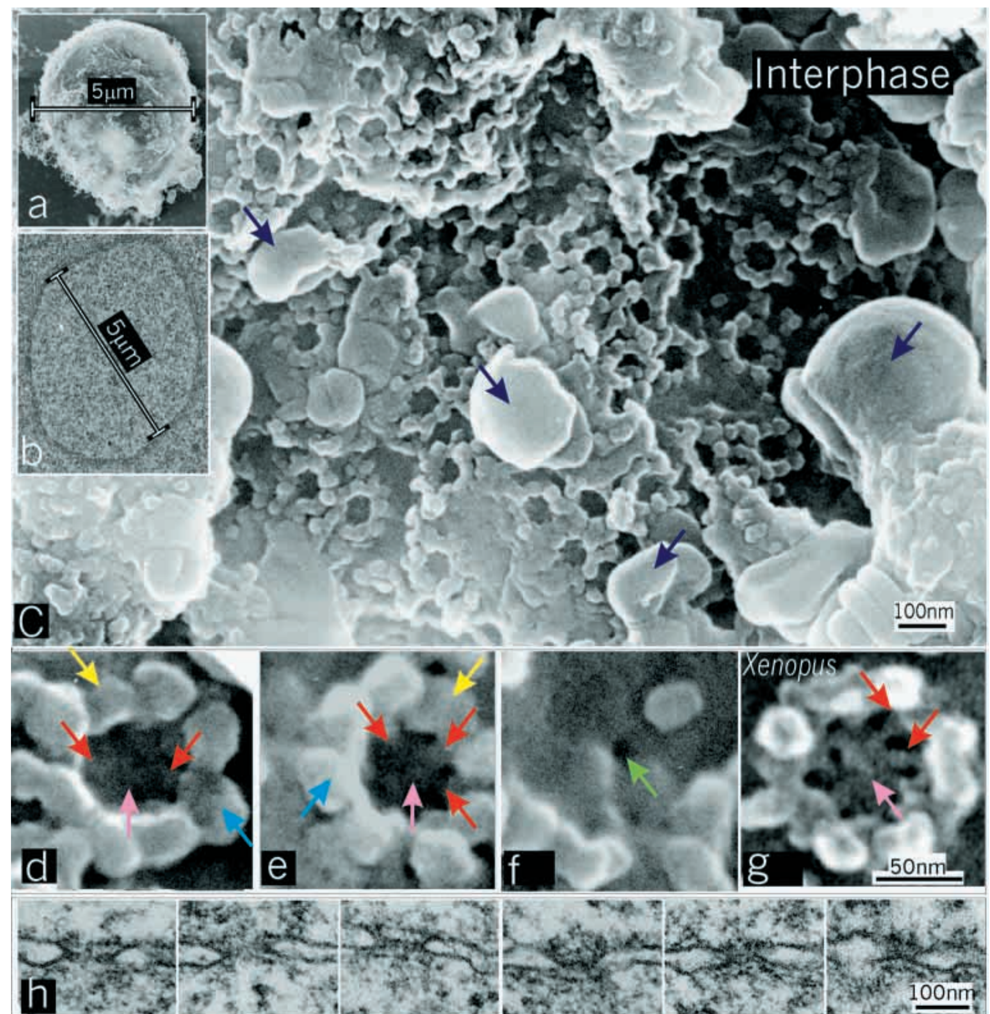

Fig. 1. Interphase nuclear morphology. (a) Nuclei vary in diameter from ~4 μm in early interphase up to ~9 μm at prophase, (b) chromatin is generally dispersed and (c) there are membrane protrusions (dark blue arrows) and numerous NPCs (d,e), which have a morphology that is similar to other higher eukaryotes such as *Xenopus* (g), containing a cytoplasmic ring (yellow arrows), cytoplasmic particles (light blue arrows), internal filaments (red arrows) and a central structure (pink arrows). (h) Cross sections of interphase NPCs showing typical NE profiles.

Interphase NPCs have a similar structure to *Xenopus* oocyte NPCs

In mid-interphase, most NPCs have a structure (Fig. 1) that is consistent with previously published FESEM images of NPCs (Goldberg and Allen, 1996; Kiseleva et al., 1998; Goldberg et al., 1997a). They have a diameter of ~110 nm, a cytoplasmic ring consisting of eight subunits (Fig. 1d, yellow arrows), upon which are attached 20 nm cytoplasmic particles (Fig. 1d,e, blue arrows). The centre of the structure of mature NPCs is always filled with material, which is preserved or resolved to differing degrees. Sometimes, it appears as a diffuse mass (Fig. 1c), but never as an empty hole and, at high magnification, some details can sometimes be discerned (Fig. 1d,e). This appears to be consistent with our previously published images of *Xenopus* oocyte NPCs. Fig. 1g shows a typical example of a *Xenopus* NPC, in which there is a central mass (pink arrow) at the level of the cytoplasmic ring with radiating filaments (red arrows) that attach this mass to the cytoplasmic ring subunits. These filaments were termed the internal filaments, which we described previously in *Xenopus* (Goldberg and Allen, 1996), *Chironomus* (Kiseleva et al., 1998), birds (Goldberg et al., 1997b) and fish (Allen et al., 1998), and we believe they attach to the top of the transporter. Images of *Drosophila* NPCs show what appears to be a similar central mass (Fig. 1d,f, pink arrows) and we see evidence that is suggestive of the radiating internal filaments (red arrows). It should be noted that the central transporter remains a controversial structure and the images presented here do not confirm its existence beyond what has been previously shown. The central material could be interpreted as material moving through the central channel (e.g. transport complexes or possibly mobile NPC components), which would account for its variable appearance. The variability could also be due to the structural dynamics of the transporter (Akey, 1990; Kiseleva et al., 1998).

At this stage there are also a few structures that are consistent with assembly intermediates (Fig. 1f, green arrow shows a ‘dimple’), suggesting that NPC assembly continues through much of the cell cycle. It also shows that our isolation and fixation protocol preserves complete NPCs alongside incomplete ones, suggesting that the incomplete NPCs we observe are unlikely to be damaged or badly preserved ones. There is also a high density of NPCs, with each nucleus containing ~2500.

There are several membrane structures attached to the outer nuclear membrane (Fig. 1c, arrows), which could be either vesicles involved in NE growth or remnants of the endoplasmic reticulum.

Cross sections of an interphase nucleus and NPCs are shown in Fig. 1b,h, showing typical NPC profiles, consistent with FESEM observations.

Prophase

We define early prophase nuclei as those that have attained a diameter of ~9 μm (Fig. 2a), that have evidence of NPC disassembly and whose chromosomes have not yet condensed. In TEM images, the evidence for NPC disassembly is a loss of electron density from the nuclear pore (Fig. 2c, arrows, compared with Fig. 1h), whereas, in FESEM images, we observe the appearance, more or less synchronously, of structures that we previously identified as assembly intermediates in *Xenopus* egg extracts. In particular we see star rings, which would be exposed by removal of the cytoplasmic ring (Goldberg and Allen, 1996) (Fig. 2b,d-f). At high magnification, the centre of the structure appears as an ‘empty’ 20-40nm hole (Fig. 2d-f, arrows), suggesting that the transporter or central material has been released together with cytoplasmic ring subunits. In some cases, we can observe a ring within the central channel that

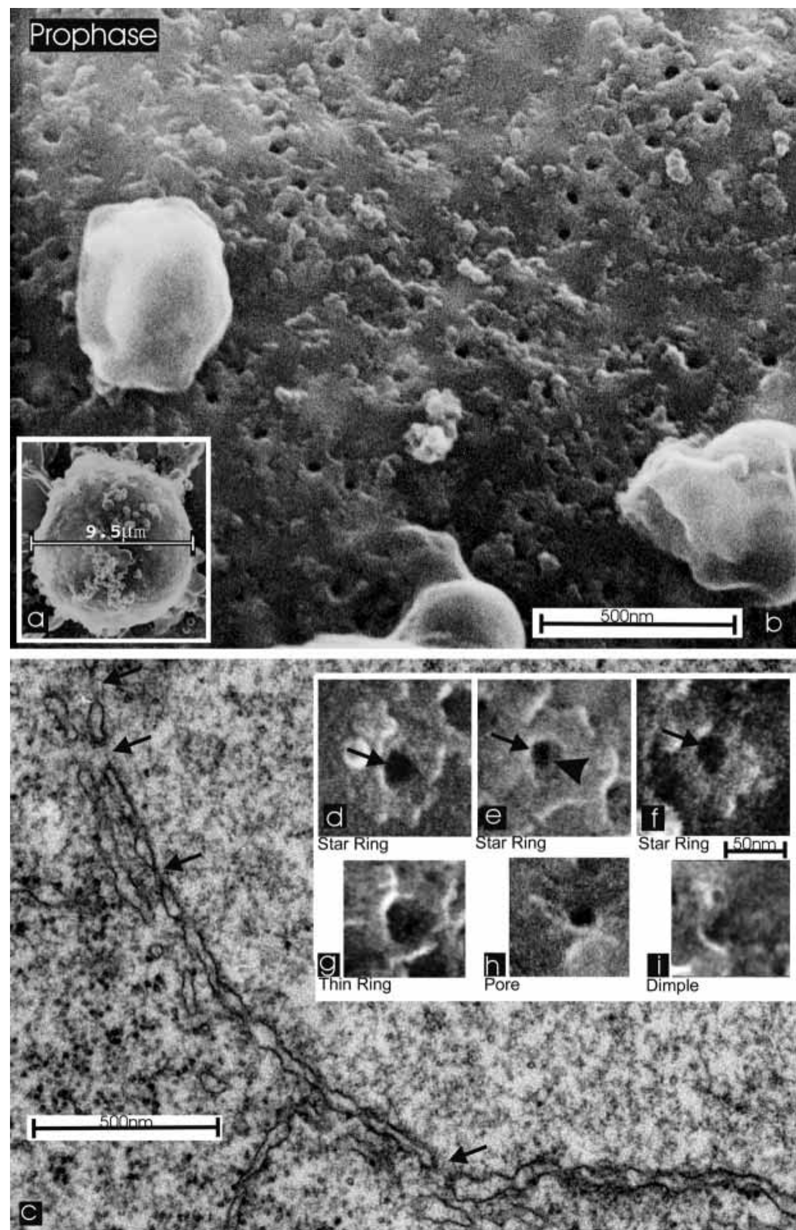

Fig. 2. Prophase nuclear morphology. (a) Nuclei are spherical with ~9.5 μm diameter. (b) NPCs appear to be partially dismantled and (c) the central channel is less electron dense in TEM thin sections (arrows). (d-f) FESEM also shows that they are empty in the middle, suggesting that the transporter has been removed leaving the star ring. (g-i) Other intermediates observed in prophase.

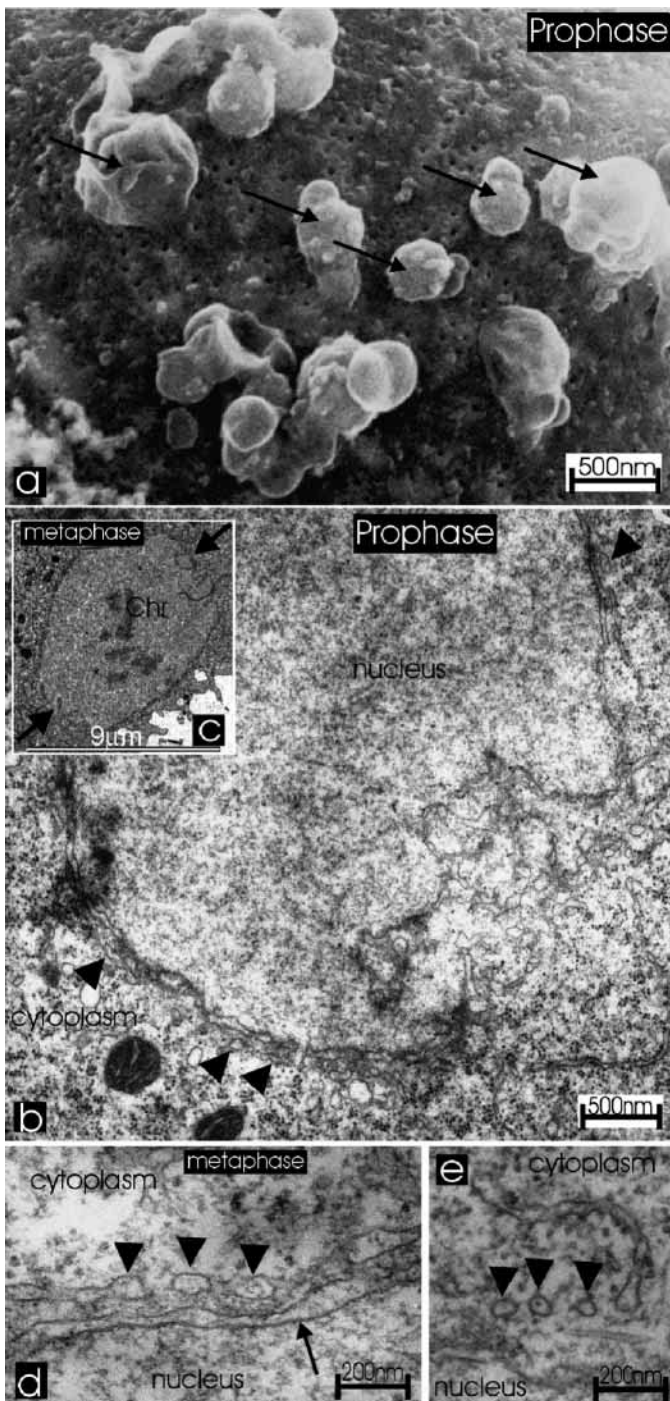

Fig. 3. Prophase/metaphase nuclear morphology. (a) FESEM image of prophase nucleus where vesicles are found at defined regions on the nuclear surface (arrows), presumably the nuclear poles, which are about to disrupt. (b) TEM thin section of more advanced stage of polar disruption in late prophase, showing membranes accumulating on the surface of the NE (arrowheads). (c) Low magnification TEM image showing disruption of poles (arrows) and penetration of the spindle microtubules in metaphase. (d,e) Mitotic NEs showing accumulated spindle membranes near or around the NE (arrowheads).

might be the inner spoke ring (Fig. 2e, arrowhead). At this stage, we also see a few other intermediates, such as pores and dimples (Fig. 2h,i).

From this, we conclude, in agreement with others (Stafstrom and Staehelin, 1984), that mitotic disassembly occurs in loose synchrony via a series of structural intermediates that are also observed during assembly (Goldberg et al., 1997a). Disassembly starts with release of the central transporter and removal of the cytoplasmic ring subunits, which are peripheral membrane components, before removal of the star ring, which appears to persist longer, possibly because it has integral membrane components (Goldberg and Allen, 1996). Finally, the remaining 40 nm diameter pore decreases in size to 20 nm and then possibly closes up.

Metaphase

In *Drosophila* early embryos, in which mitosis is completed rapidly, the chromosomes remain enclosed within the NE except at the poles. TEM (Fig. 3c) shows that NPC disassembly is probably complete, leaving an apparently continuous double membrane around the chromosomes (Fig. 3d, arrow), except at the poles adjacent to the centrioles, where the spindles access the chromosomes. In prophase, we observe the beginnings of the polar disruption of the NE (Fig. 3a,b), which occurs before the complete disassembly of the NPCs in metaphase. There is a localized formation of vesicles at the nuclear surface at each pole of the nucleus (Fig. 3a, arrows). This is accompanied by an accumulation of loosely associated vesicles and/or tubules at the surface of the non-polar region of the mitotic NE, which are maintained throughout metaphase (Fig. 3d,e, arrowheads). These vesicles/tubules are 200-300 nm in diameter and might contain NE markers such as otefin and lamins (Harel et al., 1989), suggesting that they are nuclear membrane precursors, poised to be reinserted during the rebuilding of the interphase NE.

Early telophase

We know from TEM (Fig. 3c,d) that the NPCs are probably completely dismantled during metaphase, because we never see profiles of NPCs or partially dismantled NPCs at this stage. We cannot, however, rule out the possibility that small intermediates such as dimples of 5-15 nm diameter persist, because these might not be detectable in the 50-70 nm depth of TEM sections. Therefore, the NPC-like structures that we see in early telophase are likely to be *de novo* formed NPC assembly intermediates. At this stage (Fig. 4), which is early in the reformation process, we see structures that we previously predicted were early assembly intermediates. Initially, these are low in number (there are approximately six NPCs per μm^2 in early telophase, compared with ~12 in interphase-prophase), supporting the suggestion that the holes observed in prophase do indeed close up during metaphase. The intermediates include dimples in the outer nuclear membrane, 'stabilized' holes (Fig. 4b, arrows) and some star rings (Fig. 4e, arrows). By contrast, in late telophase (Fig. 5), we see many star rings and a few NPC structures with incomplete cytoplasmic rings (a later intermediate) and, in early interphase (Fig. 7), there are many mature NPCs. This temporal appearance of these structures supports the model that there is a sequential progression from early intermediates to mature NPCs.

In early telophase, we also observe ~200 nm vesicles (Fig. 4b, stars) or membrane networks (Fig. 4d,e) that appear to have fused

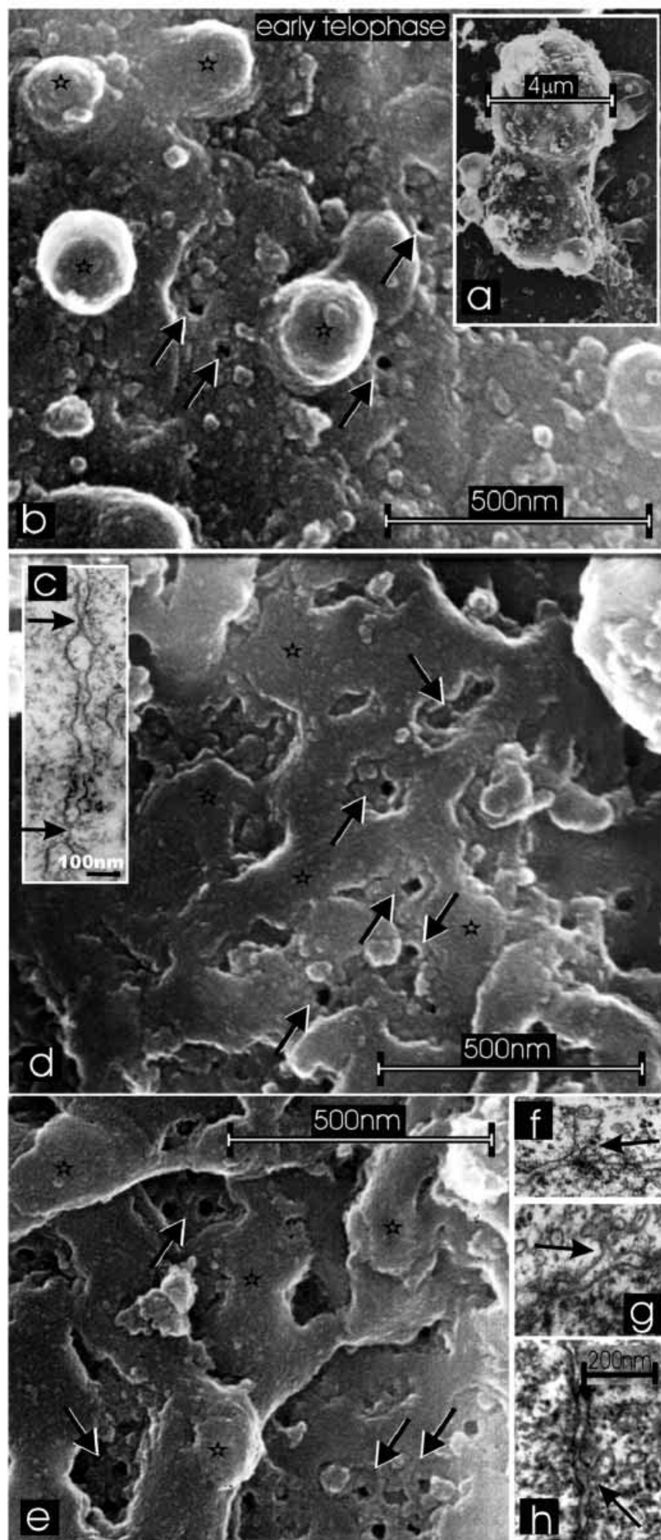

Fig. 4. Early telophase nuclear morphology. (a) Low magnification FESEM image showing bilobal morphology of a nucleus dividing into two 4 μm 'daughters'. (b) Surface of such a nucleus showing a few early assembly intermediates (arrows) and bound membrane vesicles (stars), which could be spindle membranes. (c) TEM thin section showing reassembling NPCs. (d,e) Network of spindle envelope overlying the NE (stars) and early NPC intermediates (arrows) assembling on the NE between the spindle envelope tubules. (f-h) Thin section TEM showing fusion of spindle membrane with NE (arrows).

with the outer membrane. Presumably, these are the membranes, possibly derived from the polar regions, that are observed associated with the membrane during metaphase. This membrane fusion is also seen in cross sections (Fig. 4f-h, arrows).

Late telophase

In late telophase (Fig. 5), the predominant NPC intermediate is the star ring (Fig. 5i-m) and stabilized holes are also observed (Fig. 5e-g). We have also observed a novel structure that has a 'rosette'-like appearance (Fig. 5a,b, circled; Figs 5h, 7c, arrows). We speculate that this is a further intermediate structure, possibly forming part of the inner spoke ring (based on its size, position and morphology). The fact that it is smaller than the star ring suggests that the rosette is assembled prior to the star ring. The rosette is, however, a rare structure, making it difficult to quantify and therefore we cannot exclude the possibility that it is a malformed or damaged NPC-like structure. As with all the intermediates, it remains to be proved by immuno-gold labelling that these structures contain nucleoporins and are thus NPC precursors. However, gold-conjugated antibodies used to label the structures obscure the details of the structure, making it difficult to identify components of the intermediates. Nevertheless, the rosette is an NE structure with up to eight components in a circular arrangement with a size smaller than an NPC, which are all suggestive of an NPC assembly intermediate.

Interestingly, most NPC intermediates observed at early and late telophase are localized at the membrane folds and appear initially to form in furrows where the overlying membrane sheet or vesicles fuse with the outer nuclear membrane (Fig. 4d, arrows). This fusion of the membrane sheet with the outer membrane causes an excess of membrane, creating a highly convoluted surface with the outer membrane 'billowing out' between the NPCs. This is also seen in late telophase (Fig. 5a,b), when incorporation of the membrane sheet is more advanced, but nascent NPCs are seen assembling in the highly curved regions at the junction between the outer nuclear membrane and the overlying spindle membrane (Fig. 5a, arrows). We therefore speculate that this junction is a favourable position for NPC assembly. NPCs assemble in clusters, first at the sites of fusion between membrane sheets or vesicles (Fig. 6a,d), then, as the membrane vesicles/sheets are dispersed into the NE, the nascent NPCs appear to be left in a semicircular configuration (Fig. 6b). This results in NPC clusters in early interphase that are then dispersed, probably as the lamina assembles (Paddy et al., 1996).

Early interphase

In early interphase, there are mature NPCs, thin rings (Fig. 7, large arrows) and some other intermediates (Fig. 7, small arrows). NPCs are partially clustered and there is evidence of some excess outer nuclear membrane as NPC-free areas, the borders of which seem to be where the earliest intermediates are located.

Quantification of intermediates at each stage of mitosis

Our model predicts the following order of assembly of intermediates in vivo: dimples → pores → (rosettes →) star

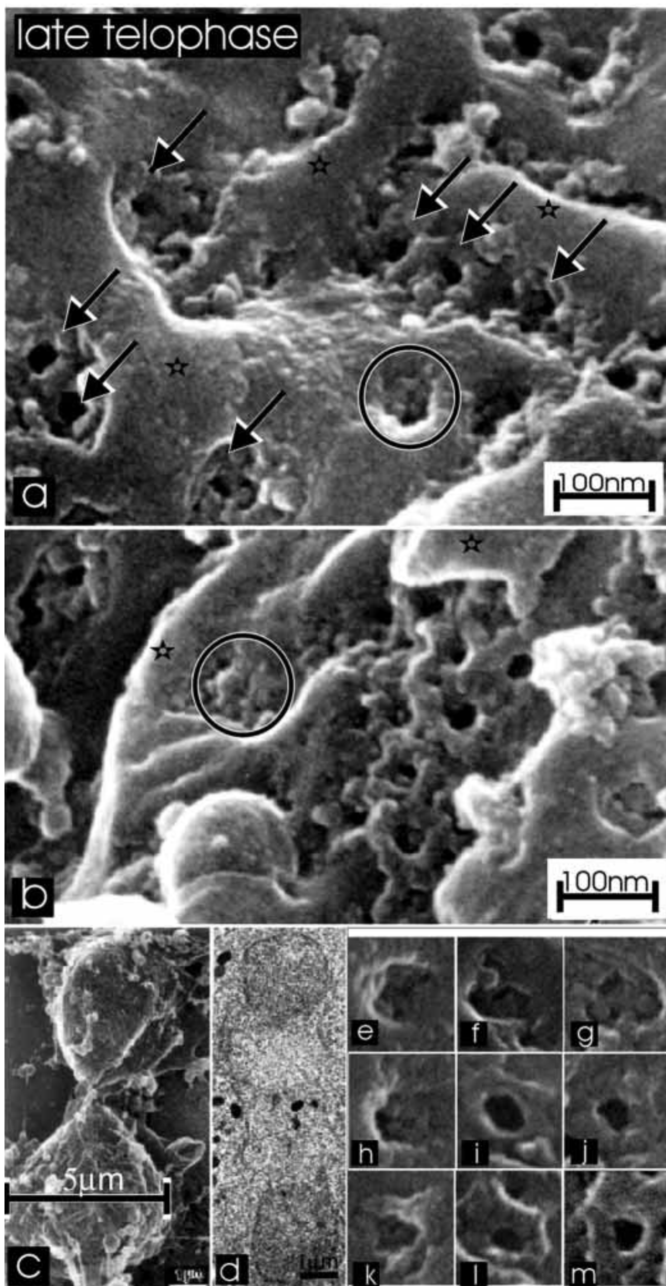

Fig. 5. Late telophase nuclear morphology. (c) FESEM of almost separated 'daughter' nuclei, (d) also shown in thin section. (a,b) Spindle membrane (stars) has fused with NE and appears to be flattening into it. NPCs form at the junction between the spindle envelope and NE (arrows), and we also observe a novel 'rosette' structure (circled). (e-m) Montage of selected intermediates at this stage. (e-g) Stabilized pores with structures, probably the beginnings of the spoke ring complex, assembled into central channel. (h) Rosette structure. (i-m) Star rings.

rings → thin rings → mature NPCs. From this, we would suggest that early intermediates predominate in early telophase, with the number of later intermediates increasing as nuclear assembly precedes. Likewise, during nuclear disassembly, the early assembly intermediates will predominate at the later stages of prophase, whereas mature NPCs should predominate during interphase. To test this, we quantified the relative number of each intermediate as the nuclei progressed through each stage of mitosis as defined above (Fig. 8). We found that most intermediates could be detected during interphase but mature NPCs were by far the most numerous (Fig. 8a). In prophase (Fig. 8b), intermediates began to increase but this was only really very clear for the star ring (Fig 2a), although mature NPCs disappeared completely. Therefore, for disassembly, only one intermediate time point has been observed, at which the star ring, devoid of cytoplasmic rings and central structures (transporter and internal filaments) is predominantly observed. Other intermediates are observed and, as there is not likely to be any NPC assembly at this stage, they are most probably disassembly intermediates. However, the numbers are low and they could be incomplete NPCs left over from interphase.

TEM evidence suggests that NPCs are completely disassembled in metaphase, as the nuclear envelope appears as two continuous parallel lines with no joins between them (Fig. 3d), so we suggest that, at this stage, there might be no intermediates. However, we cannot rule out the persistence of early intermediates that are be to small to detect in 50-100 nm thick sections (as some of the intermediates are smaller than this).

In early telophase dimples and pores significantly predominate (Fig. 8d), whereas, in late telophase, these decrease and star rings predominate (Fig. 8e); as predicted, in early interphase star rings decrease and thin rings appear (Fig. 8f). Mature NPCs also appear at this stage and quickly increase in number to become the predominant structure again. The rosette was only found in low numbers and, without further analysis, we cannot place it with confidence in this time-ordered progression. However, based on our previous criteria of size and complexity, it would be tentatively placed between the pore and the star ring, and either represent some component of the spoke assembly or an early form of the star ring.

Statistical analysis clearly suggests a progression over time from the smaller, less complex structures to the larger, more complex ones as the NE proceeds through post-mitotic reassembly and provides evidence for our previous predictions, which are crucial to the model.

Model for NPC assembly and disassembly in vivo

Fig. 9 is a montage of the NPC assembly intermediates that we have observed in vivo. Whereas, previously, we simply arranged these into an order of increasing size and complexity (Goldberg et al., 1997a), we have here been able to provide evidence for a temporal progression between intermediates. We have also found that NPCs assemble in telophase at what appear to be inter-membrane junctions, suggesting that two membrane domains that are separated at mitosis might be required for NPC assembly.

To this, we can add a model for the mitotic disassembly of the NPC. From the evidence presented here, we propose that disassembly is, to some extent, the reverse of the assembly process. The star ring is the predominant intermediate during prophase, whereas the other intermediates are only observed in low numbers. This suggests that the initial removal of the central transporter and cytoplasmic ring subunits is rapid, whereas the star ring is more persistent. This might suggest that disassembly is a more rapid process than assembly or, as appears to be the case, that it is more synchronous, so we are

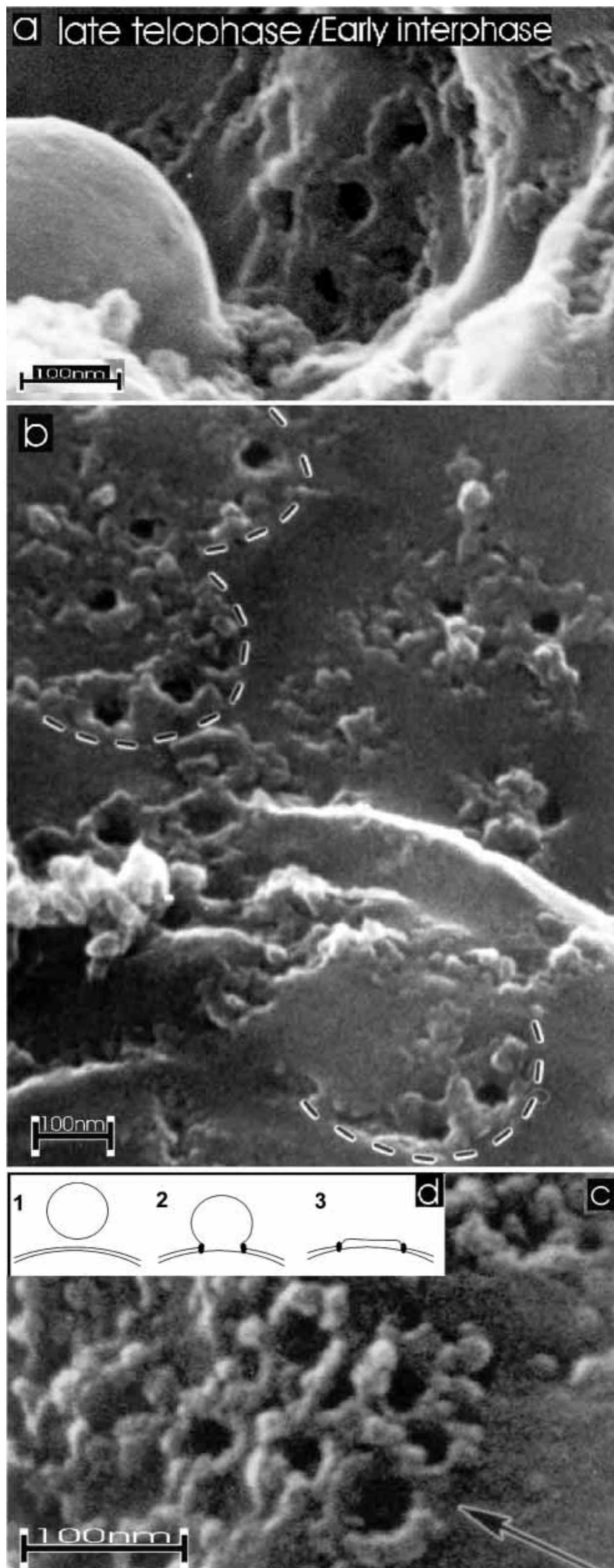

Fig. 6. Telophase/early interphase nucleus. (a) NPCs assemble at the base of bound vesicles. (b) After dispersal of the spindle membrane into the nuclear envelope, the nascent NPCs appear to be left in a semicircular arrangement. (c) NPC clusters are also observed. (d) The assembly of NPCs at the fusion junction between NE and spindle envelope or vesicles: (1) apposition of spindle vesicle to NE; (2) vesicle-NE fusion, initiating NPC assembly at the site of fusion; (3) further incorporation of vesicle membrane into NE.

more likely to miss certain transient intermediates. We cannot be certain that disassembly precisely mirrors assembly because fewer stages have been observed. However, we can say that the cytoplasmic ring and central structures (Fig. 2b) are removed before the star ring. The star ring is then removed and the pore closes up.

DISCUSSION

Using FESEM and TEM, we have studied NE structure as it proceeds through mitosis during the rapid cell cycles of the pre-blastoderm *Drosophila* embryo. For the first time, we have shown that NPC assembly and disassembly in vivo occurs through a series of intermediates that were first identified in in-vitro-assembly experiments (Goldberg et al., 1997a). We have also shown for the first time that different intermediates predominate at different stages of mitosis, providing evidence for a time-ordered progression from one intermediate to the next, as predicted in our original model. We have visualized the formation of vesicles at the nuclear poles during prophase, which we presume leads to the rupturing of the NE in these regions. We have found that, in telophase, new NPCs appear to assemble along the regions of fusion between the existing nuclear membrane and vesicles or membrane sheets fusing with it.

Model for NPC assembly and disassembly

In our previous model (Goldberg et al., 1997a), we proposed that NPC assembly starts with invagination of one or both nuclear membranes that is seen as a dimple in the outer membrane. When the two membranes contact each other, they fuse to form a pore. This is stabilized by an electron-dense ring that might be part of the spoke complex, and central material is inserted to 'plug' the pore. The star ring is then assembled, with the cytoplasmic ring being built up on top of it, and peripheral filaments are added. Here, we have seen the same intermediates but, because the size and morphology of the nuclei are diagnostic of the stage of mitosis, we have been able to show a temporal appearance of these intermediates as predicted by our model. Because the same intermediates have been observed in prophase, we would like to propose that disassembly is the reverse process to assembly, proceeding through the same structural intermediates in the reverse order. However, as only one intermediate time point has been observed (prophase), we cannot be so sure of the temporal progression, other than that complete disassembly is via the star ring, which appears to be preceded by removal of the cytoplasmic ring and filaments and the central material or transporter. Disassembly appears to be more synchronous than assembly. This would be expected because the triggering of mitosis and NE breakdown is a rapid, almost sudden, switch regulated by a positive feedback loop leading to the phosphorylation of nucleoporins (Macaulay et al., 1995). The order of disassembly of particular substructures probably reflects their accessibility to mitotic kinases, with the internal structures only becoming accessible when more peripheral structures have been solubilized.

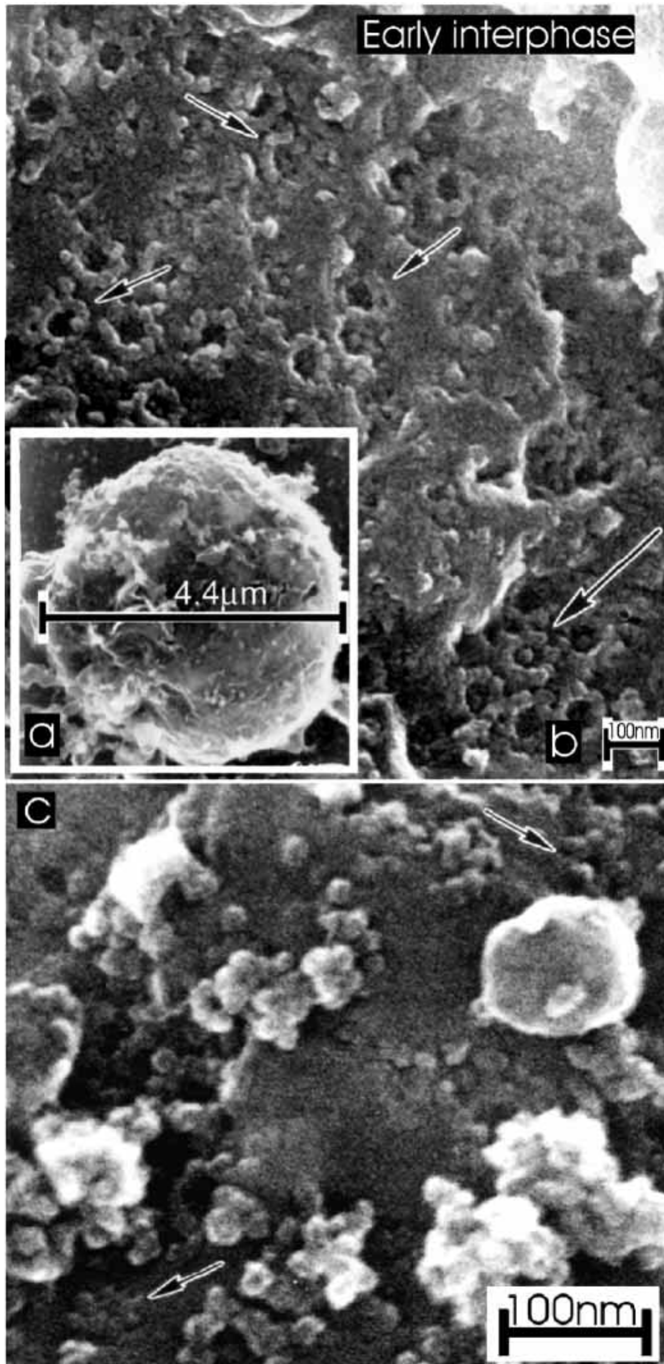

Fig. 7. Early interphase nucleus. (a) Nuclei are relatively small, (b) have mature NPCs (large arrow) but also many intermediates (small arrows), including (c) rosettes (arrows).

Assembly, by contrast, requires not only dephosphorylation of nucleoporins but also recruitment of all the dispersed components into the assembling structure in the correct order.

NPCs appear to assemble at the junction between spindle membrane and mitotic NE membrane

Although the steps of NPC assembly appear to be the same in early *Drosophila* embryos as in *Xenopus* egg extracts, the involvement of membranes in the process of NE assembly is not identical. This is unsurprising because nuclear assembly in extracts starts with the binding of membrane vesicles to naked chromatin (Lohka and Masui, 1984), whereas, in *Drosophila* embryos, the chromosomes are mostly already enclosed in a double membrane. It was previously shown that the mitotic 'nucleus' is surrounded by a second double membrane, the 'spindle membrane' (Stafstrom and Staehelin, 1984), which is probably derived from the interphase NE during spindle formation, because it contains the inner membrane proteins otefin and lamin (Harel et al., 1989).

In early prophase, we have observed by TEM an overlying membrane that is likely to be the spindle membrane. The spindle membrane appears to fuse with the underlying mitotic NE and, interestingly, NPCs appear to assemble at the junction between the NE and the spindle membrane. One explanation for this positional assembly is that both the NE and the spindle envelope are required for NPC assembly. This could be because some integral membrane nucleoporins are sorted to one membrane, whereas others are sorted to the other and it might then be necessary for the two membranes to fuse before all components required for the initial stages of NPC assembly can be brought together. It has been shown, in both *Xenopus* egg extracts (Drummond et al., 1999; Vigers and Lohka, 1991) and tissue culture (Chaudhary and Courvalin, 1993), that nuclear membrane markers might be sorted during NE breakdown into vesicles or, possibly, domains that have different roles in the assembly process.

Previously, it was shown that the lamin B receptor is redistributed to the tubular endoplasmic reticulum network during mitosis (Ellenberg et al., 1997; Yang et al., 1997), suggesting that nuclear membrane vesiculation is not a normal process in NE breakdown but rather that the nuclear membranes are 'fed' into the ER. Our observations in *Drosophila* might be consistent with the idea that membranes are sorted into domains during breakdown (if both spindle and NE membranes are required for NPC assembly) but are not released into the cytoplasm as vesicles or fed into the ER, but rather remain associated with the nucleus. It is therefore possible that the semi-closed mitosis of early *Drosophila* embryos is, in fact, not as unusual as it looks. It could be that the main difference is that the membrane domains remain in close proximity to the chromosomes and to each other rather than being dispersed into the cytoplasm or the ER. This would then allow a rapid but controlled reassembly of the NPCs in telophase, consistent with the very rapid mitoses at this stage of development.

A further explanation is that NPCs only assemble on regions of the NE that are tightly attached to the underlying chromatin, which happens to be adjacent to the fusing spindle membranes. It could therefore be that the reasons for these observations are simply topological – NPCs cannot form on the spindle membrane because they could not be transferred to the nuclear envelope as they span a double membrane, and NPCs cannot form on the underlying nuclear membrane because it is inaccessible to cytosolic components (e.g. solubilized nucleoporins). It could be that only as the two membranes fuse and attach to the chromatin do they become topologically favourable for NPC assembly. Notice that, even though chromatin is not required for NPC assembly, NPCs are preferentially assembled on chromatin-associated membranes (Dabauvalle et al., 1991). Significant evidence exists, however,

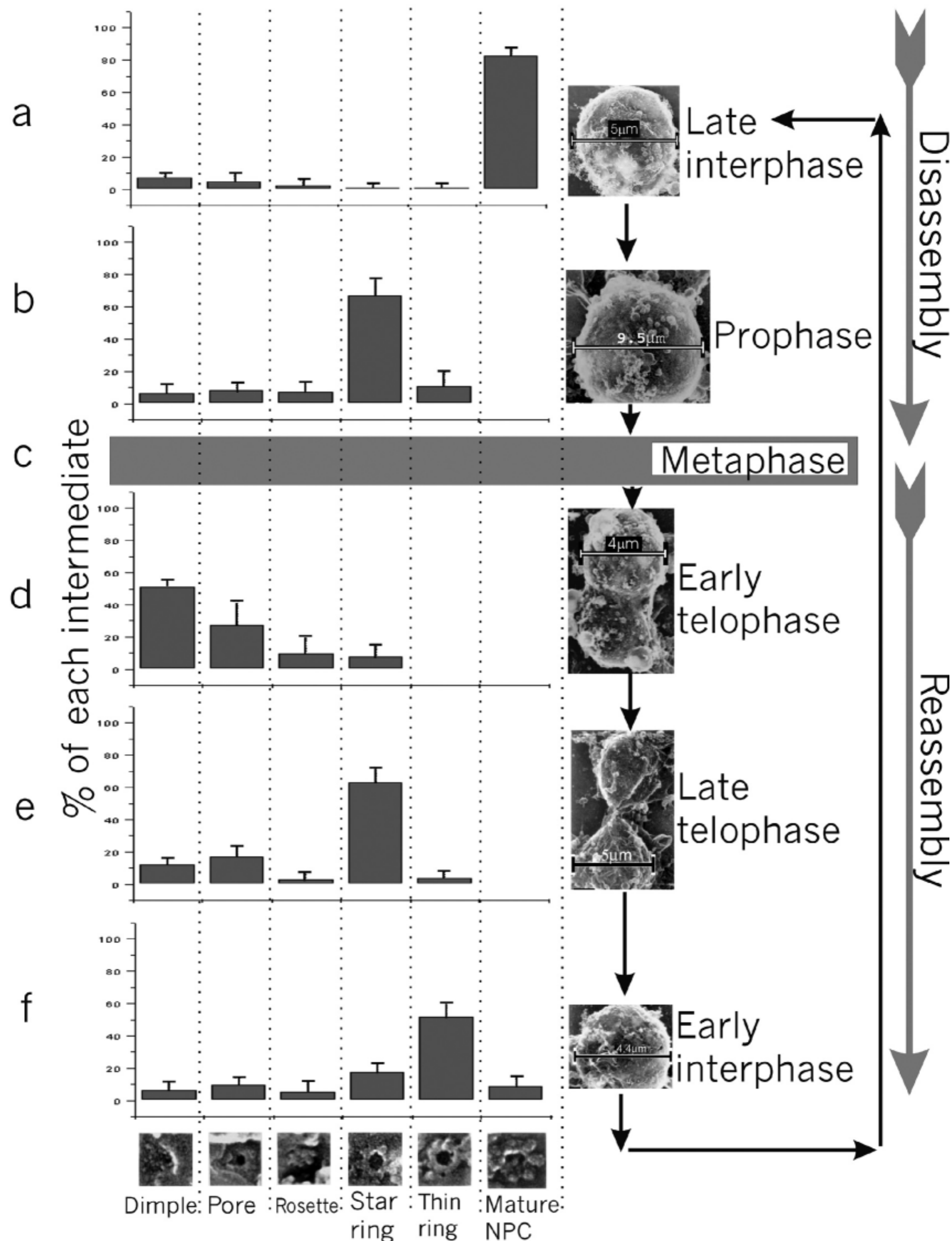

Fig. 8. Quantification of each intermediate at each stage of mitosis. Data are presented as a percentage of the total NPC-like structures at each stage. The stage of mitosis is defined on the right-hand side with an example of a nucleus used in the quantification; the intermediate structures quantified are shown along the bottom. No data is given for metaphase (c) as these could not be visualized by FESEM. This shows that mature NPCs predominate in interphase (a) but disappear rapidly in prophase (b), in which star rings predominate. In early telophase (d), early assembly intermediates (particularly dimples and pores) predominate, whereas, in late telophase (e), assembly appears to progress to star rings and then thin rings in early interphase (f).

for sorting of disassembling nuclear membranes during mitosis (Collas and Courvalin, 2000) and at least two distinct fractions can be isolated from *Xenopus* egg extracts (Vigers and Lohka, 1991; Drummond et al., 1999), both of which are necessary to initiate NE and NPC assembly. In the case of syncytial *Drosophila* embryos it has been shown that inner nuclear membrane proteins, such as lamin and otefin, are indeed sorted to the overlying spindle envelope (Harel et al., 1989). These

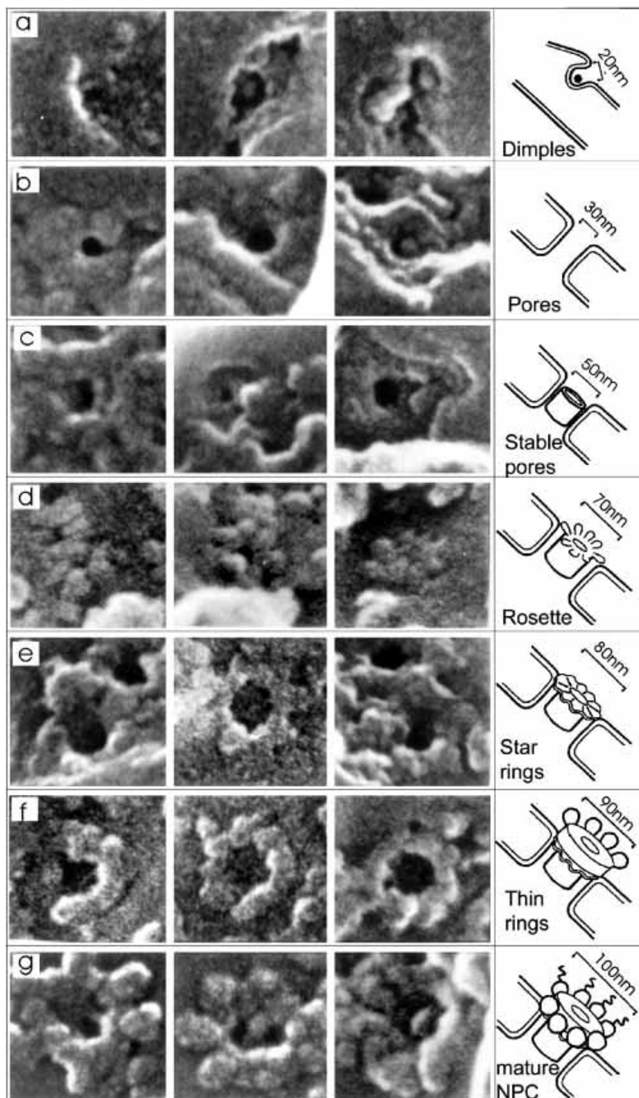

Fig. 9. Montage and corresponding illustrations of the NPC assembly intermediates (as shown in Fig. 8) observed in early *Drosophila* embryos arranged into a proposed order of assembly. Disassembly is roughly the reverse of this order

proteins might be involved in attaching the inner membrane to the chromatin, a process that could promote NPC assembly. An additional possible reason for the preferential assembly of NPCs along the sites of fusion between the NE and the spindle envelope is the negative curvature of the membrane at this point. It has been shown that negative membrane curvature stimulates recruitment of proteins (Kozlov et al., 1989) and so could cause preferential recruitment of nucleoporins to these regions. Some or all of these disparate explanations (and others) could contribute to this observation. Extensive immuno-gold labelling of thin sections through teleophase nuclei using antibodies to nucleoporins and inner membrane proteins will help to resolve this.

NPC clusters are observed in early interphase, possibly because they have assembled in specific regions. Clusters are also observed in early NE assembly in egg extracts (Goldberg et al., 1992). It has been shown that nuclei assembled in vitro without a lamina have NPCs that remain in clusters (Goldberg et al., 1995) and, in *Drosophila*, flies containing a mutated gene for lamin Dm_0 also have clustered NPCs (Lenz-Böhme et al., 1997). As the lamina is assembled relatively late in the NE reassembly process, it is likely that the lamina or lamin-associated proteins (Gotzmann and Foisner, 1999) are required to distribute the NPCs as early interphase progresses.

Summary

Because we can identify the stage of mitosis by the morphology of nuclei in the semi-closed mitosis of early *Drosophila* embryos, we can assign the appearance of NPC intermediates to specific stages of nuclear disassembly or assembly, confirming in vivo the previous in vitro model for the steps of NPC assembly. We find that NPCs do not assemble in random positions but apparently at the site of fusion between the spindle envelope and the mitotic NE. We have also shown that same structural intermediates might also be involved in NPC disassembly.

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