Visualisation of the dynamics of Nuclear Envelope Reformation in mammalian cells.

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In all higher eukaryotes, the nuclear envelope (NE) breaks down at the start of mitotic prophase, and is re-established in telophase as the chromatids of each daughter cell decondense to form interphase nuclei. This is in contrast to yeast and some insects, where the NE is retained throughout division, in a 'closed' mitosis. The series of events during NE reformation has been studied extensively in model, cell free systems, often involving amphibian egg extracts, which will reform a nuclear envelope *in vitro* around even naked DNA, but more usually, demembranated sperm chromatin. Our own studies (1) with this system have shown that vesicles from the extract which will form the new nuclear membrane attach to the surface of the sperm chromatin, spread out and fuse with adjacent vesicles to complete enclosure. At the same time nuclear pore complexes (NPCs) become inserted in the NE, developing through a well established series of intermediate levels of assembly, many of which can be experimentally modulated (1). Once enclosure is complete, the *in vitro* formed nucleus undergoes a precisely controlled DNA replication, confirming the physiological relevance of the system. In this system however, areas of novel membrane must always be present before NPCs are inserted, even if the new membrane patch is barely wide enough to accommodate the diameter of the NPC. Indeed, if NPC formation is experimentally blocked, NE formation will still be completed, allowing subsequent insertion of NPCs .

In a series of investigations to compare stages of *in vitro* NE reformation with higher eukaryotes *in vivo*, we have developed approaches to visualize NE re-assembly in whole cells, (HeLa, DLD) This approach produces access for surface imaging by FESEM *in situ* in dividing cells. We have extended the protocol for accessing interphase nuclear surfaces to cells in division (2).Briefly, this involves fixation, (10 secs in 2% paraformaldehyde,0.01% glutaraldehyde), followed by extraction in 0.5% Triton (15-30mins),which may be followed by antibody incubations, then re-fixation in 3% Glutaraldehyde and 1% Osmium, dehydration and critical point drying. The cells are then subjected to a simple 'dry-fracture' where the Si chip on which the cells were grown is touched to double sided tape. This produces fractures which vary from exposure of the upper surface of the interphase nucleus to various depths within the nucleus itself. Both sides of the fracture can be studied in the SEM. In the case of dividing cells, the fracture removes enough of the detergent resistant cytoskeletal remains to allow direct imaging of the chromosomal surfaces. Specimens are coated with 2nms of Cr, which does not inhibit the BSE signal from either 10 nm or 5 nm Au colloid marking the secondary antigen sites. Condensed chromatin after osmium fixation itself generates a strong BSE signal, but the Au label still stands out in the BSE image. We have shown 10nm Au particles at low magnifications of 8-10,000X, which is useful for demonstrating the overall distribution across the cell. Our system of signal acquisition is to optimize the SE and BSE signals separately, and then acquire each simultaneously (on the same scan) at 2800 X 2000 pixels. This ensures that the register between SE and BSE images is maintained, for exact superimposition of images

 HeLa cells were accessed as above in successive stages of mitosis, and imaged with immunostaining for mAb 414 (an antibody which binds the O –linked Glyc-Nac sugar residues common to several nucleporins), and also for individual members of the 107 -160 Nup protein

complex which binds early in NE reformation and mediates NPC assembly.(3).In prophase and metaphase the chromosomes were largely unlabelled, but in anaphase and telophase, labeling was widespread, both for Nup107 and Nup133 mAbs and also mAb 414.Surprisingly ,we could recognize several stages of NPC formation in process directly on the surface of the chromatin, in the complete absence of new NE membrane. This is in direct contrast to our findings in NPC formation *in vitro,* where new NPC formation appears to require the presence of an area of flattened membranes in access of the diameter of a NPC to allow membrane fusion as an initial event in NPC formation(1).
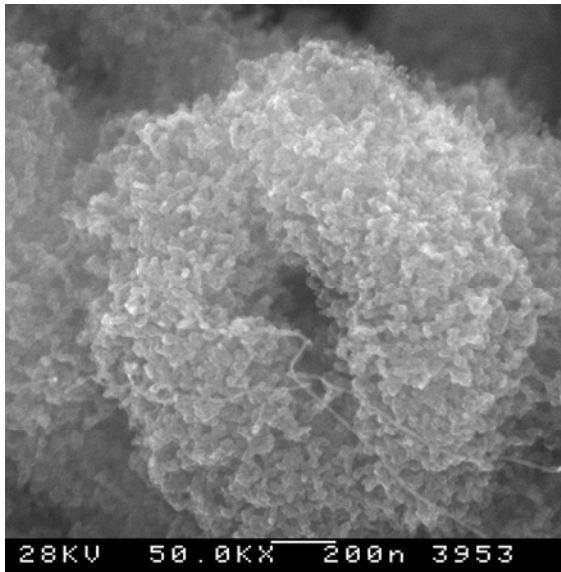
Fig 1. Chromosome surface appearance at prophase is shown to consist of a series of loops of 30nms chromatin fibres.
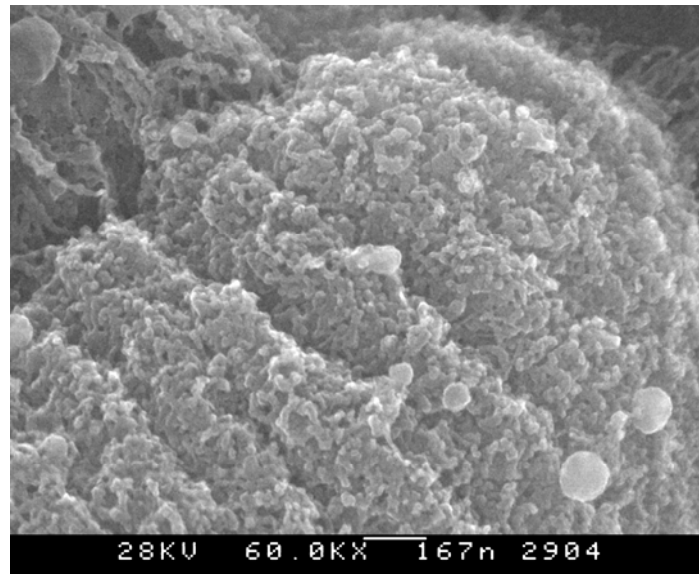
Fig 2. The decondensing chromatids at anaphase show circular profiles of newly forming NPCs directly on the chromatin surface.

These findings suggest a novel method for the formation of NPCs which appears to take place at the latter stages of mitosis, where nucleoporin protein complexes such as the 107-160 complex, bind directly to the surface of the chromatin, as indicated in Walther et al (2003).These complexes must then provide sites for further nucleoporins to bind and initiate further progression towards complete NPC assembly in the absence of NE membranes at this stage. Similar findings were reported in *in vitro* nuclear formation experiments some time ago by TEM, where unusual profiles were observed at the surface of the chromatin, during *in vitro* nuclear formation in conditions in which membrane precursors were severely reduced. These structures were termed prepores.(4) The findings reported here suggest that prepores may well exist *in vivo*, as a post mitotic mechanism, although once enclosure of new daughter nuclei has occurred, new NPCs need to be inserted into existing NE membranes as suggested previously (1).

References:
Goldberg,M.W. et al, **J.Cell Science** 110,(1997) 409-
Allen T.D.et al, **Methods in Cell Biology** 53, (1998) 125-
Walther T.C. et al, **Cell** , 113(2003) !95-
Sheehan M.A. et al **J.Cell Biol.**,(1988) 1-