Live cell imaging and electron microscopy reveal dynamic processes of BAF-directed nuclear envelope assembly

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Summary
Assembly of the nuclear envelope (NE) in telophase is essential for higher eukaryotic cells to re-establish a functional nucleus. Time-lapse, FRAP and FRET analyses in human cells showed that barrier-to-autointegration factor (BAF), a DNA-binding protein, assembled first at the distinct ‘core’ region of the telophase chromosome and formed an immobile complex by directly binding with other core-localizing NE proteins, such as lamin A and emerin. Correlative light and electron microscopy after live cell imaging, further showed that BAF formed an electron-dense structure on the chromosome surface of the core, close to spindle microtubules (MTs) prior to the attachment of precursor NE membranes, suggesting that MTs may mediate core assembly of BAF. Disruption of the spindle MTs consistently abolished BAF accumulation at the core. In addition, RNAi of BAF eliminated the core assembly of lamin A and emerin, caused abnormal cytoplasmic accumulation of precursor nuclear membranes and resulted in a significant delay of NE assembly. These results suggest that the MT-mediated BAF accumulation at the core facilitates NE assembly at the end of mitosis.

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Key words: Barrier-to-autointegration factor, Chromatin, Emerin, Lamin A, Nuclear envelope, Microtubule

Introduction
The nuclear envelope (NE) is a cellular structure that encloses chromosomes and provides a physicochemical framework for genetic activities such as gene expression and DNA replication. The NE is a dynamic structure in eukaryotes, disassembling at prophase and reassembling at telophase in each mitosis. The reassembly of the NE is crucial to re-establish a functional nucleus for the next interphase and to ensure proper progression of the cell cycle. Several lines of evidence have shown that defects in NE architecture can cause diseases in animals. Recently, a variety of human disorders known as ‘nuclear envelopathy’ or ‘nuclear laminopathy’ have been reported (reviewed by Nagano and Arahata, 2000; Wilson et al., 2001; Worman and Courvalin, 2005). These disorders involve mutations in NE components. Mutations in the gene encoding lamin A (official protein symbol, LMNA) cause 12 human diseases (reviewed by Mattout et al., 2005) including Emery-Dreifuss muscular dystrophy (Bonfante et al., 1999; Clements et al., 2000) and Hutchinson-Gilford progeria syndrome (Eriksson et al., 2003; Mounkes et al., 2003). Emery-Dreifuss muscular dystrophy can also be caused by mutations of the gene encoding emerin (offical protein symbol, EMD) (Bione et al., 1994). The fact that defects in the NE can cause many different diseases indicates that the NE is involved in a wide variety of cellular functions. Thus, understanding the dynamic architecture of the NE will provide insight into the regulatory mechanisms for these cellular functions.

Molecular mechanisms of NE formation have been well described in vitro experimental systems (Hetzer et al., 2000; Harel et al., 2003; Hachet et al., 2004); however, compared with NE formation in vitro, NE reformation in vivo is poorly understood. It is believed that it begins with attachment of precursor membranes to telophase chromosomes, followed by fusion of the membranes and reassembly of integral membrane proteins, the NPC, and nuclear lamina into the NE. Chromatin-binding integral membrane proteins, such as lamin B receptor (LBR) and LAP2β (official symbol, LAP2B) are proposed to possess a role in early assembly of the NE (Gant and Wilson, 1997; Ulbert et al., 2006). Assembly of LEM-domain NE proteins such as emerin and LAP2β depends on barrier-to-autointegration factor (BAF), a chromatin-binding protein, in human cells (Haraguchi et al., 2001).

BAF forms dimers that bind nonspecifically to double-stranded DNA in vitro (Lee and Cragie, 1998) and can be phosphorylated in its N-terminal region by the vaccinia-related kinases (Nichols et al., 2006; Gorjánácz et al., 2007). BAF was first discovered as a cellular protein that prevents retroviral DNA from suicidal auto-integration and ensures its integration into host DNA (Lee and Cragie, 1998; Jacque and Stevenson, 2006). It was later described as binding partner of LEM-domain NE proteins (Furukawa, 1999; Lee et al., 2001; Shumaker et al., 2001). BAF is proposed to have a function in NE assembly in human cells (Haraguchi et al., 2000) and C. elegans (Margalit et al., 2005; Gorjánácz et al., 2007).
In human cells, BAF accumulates with emerin, at the central regions of the assembling nuclear rim close to the spindle microtubules (MTs) during telophase when the NE is reforming. This region is named the ‘core’ region (Haraguchi et al., 2001). In our previous study, we found that emerin, LAP2β and lamin A accumulated in the core region during telophase in a BAF-dependent manner (Haraguchi et al., 2001). LAP2α (official symbol, LAP2A) also accumulates at the core (Dechat et al., 2004). Although the core structure seems important for NE assembly of the LEM-domain proteins and lamin A, its structural basis and dynamics in living cells are largely unknown. To understand the structural basis and biological significance of the core region, we have examined physical and chemical properties of the core region by time-lapse microscopy of living cells, FRAP and FRET analyses, and EM analysis combined with live-cell imaging. In addition, we used RNAi to selectively remove individual components of the core structure, BAF, lamin A and emerin, to examine their functions in living cells. Here we report that BAF forms an electron-dense stable structure at the core region of the telophase chromosome in a spindle-MT-dependent manner, and propose that this MT-dependent BAF accumulation at the core coordinates efficient rendezvous of nuclear membranes and chromosomes.

Results

BAF assembles first to the ‘core’ region of the reforming telophase nuclear envelope

Assembly of NE proteins at the end of mitosis is an essential step in the reformation of a functional nucleus in higher eukaryotic cells. In order to elucidate underlying molecular mechanisms of NE reformation in human cells, we examined spatial and temporal sequences of NE assembly by monitoring the behavior of NE-GFP fusion proteins in living cells. The NE proteins tested were BAF (which binds both chromatin and LEM-domain proteins); LAP2β, emerin and MAN1 (membrane-bound LEM-domain NE proteins); LAP2α (LEM-domain protein localized in the nucleus); lamin A and lamin B1 (major components of the nuclear lamina); LBR (classical integral nuclear membrane protein); and nucleoporin Nup35 (official protein symbol, NUP53). Time-lapse observation of HeLa cells expressing each one of these NE fusion proteins showed that BAF, LAP2α, LAP2β, emerin, MAN1 and lamin A accumulated at a specialized region of the telophase chromosome mass close to the spindle (the ‘core’ region; see Fig. 1A and images indicated by arrows in Fig. 1B). This accumulation occurred during a limited period of telophase, as described previously (Haraguchi et al., 2000; Haraguchi et al., 2001; Dechat et al., 2004). In contrast with these core-localizing proteins, lamin B1, LBR and Nup35 did not show clear accumulation at the core region (Fig. 1B).

The fluorescence intensity of NE proteins at the core region is plotted in Fig. 1C; the timing of core localization was determined as the time, in seconds after the metaphase-anaphase transition, at which 80% of maximum accumulation had occurred (Fig. 1D). Among the core-localizing proteins, BAF accumulated earliest at the core region, approximately 434 seconds after the metaphase-anaphase transition. LAP2α, lamin A and emerin accumulated approximately 30 seconds later, and LAP2β and MAN1 accumulated after approximately another 30 seconds (Fig. 1C,D).

That BAF accumulated first was confirmed by observation of HeLa cells expressing mRFP-BAF transiently co-transfected with GFP-emerin or GFP-lamin A. In these cells, mRFP-BAF accumulated in the core region earlier than GFP-emerin or GFP-lamin A (data not shown). These results indicate that BAF appears at the core region prior to the accumulation of other core-localizing NE proteins. Since BAF binds both emerin and lamin A in vitro (Lee et al., 2001; Holaska et al., 2003), the early accumulation of BAF at the core region suggests the possibility that BAF provides a structural foothold to allow assembly of nuclear lamins and membrane-bound NE proteins. The time course of assembly to the reforming NE at telophase is schematically summarized in Fig. 1E.

BAF forms an immobile structure at the core region

From our time-lapse analysis, we hypothesized that BAF might form a foothold on the chromosome surface to allow assembly of other NE proteins. To test this hypothesis, we examined the mobility of BAF at the ‘core’ region during telophase by FRAP analysis of HeLa cells stably expressing GFP-BAF. We previously reported that GFP-BAF during interphase was highly mobile with a half time recovery of 270 mseconds and an immobile fraction (IM) of approximately 3% (Shimi et al., 2004). GFP-BAF was also highly mobile during metaphase (data not shown). By contrast, GFP-BAF became highly immobile during telophase with an IM of 72±16% (n=7) (Fig. 2A,B), and remained immobile until the core region disappeared at the end of M phase; GFP-BAF regained its high mobility in early G1 phase (data not shown). This result indicates that physical attributes of BAF are regulated during the cell cycle, with BAF forming an immobile structure at the core region specifically in telophase, a characteristic also exhibited by LAP2α (Dechat et al., 2004).

To further examine physical properties of the core structure, we determined the mobility of other core-localizing NE proteins, such as lamin A and emerin, at the core region. These NE components showed a similar mobility to that of BAF with an IM of 74±14% (n=5) for lamin A and 46±7% for emerin (n=5) (Fig. 2C,D). These results suggest that BAF may form a stable higher-order complex together with these NE proteins at the core.

BAF directly interacts with BAF, lamin A and emerin at the core region

Because BAF, lamin A and LEM-domain NE proteins all become immobile at the core region, the core structure may consist of a higher-order complex of BAF with lamin A and the LEM domain NE proteins. To test whether BAF directly interacts with these NE proteins at the core region, we used fluorescence Förster resonance energy transfer (FRET) analysis. In HeLa cells transiently coexpressing mVenus-BAF and mCFP fusions of the second protein, FRET signals were detected by acceptor photobleaching (Fig. 3). The results showed that mVenus-BAF produced FRET signals with mCFP-BAF (Fig. 3A,B), mCFP-lamin A (Fig. 3C,D) and mCFP-emerin (Fig. 3E,F). In addition, time-lapse ratio imaging between mCFP-BAF and YFP-BAF showed that FRET signals between BAF molecules dramatically increased in the core region during telophase (Fig. 3I). As BAF forms a dimer or dodecamer in vitro (Zheng et al., 2000), this high FRET signal may represent a higher-order complex structure. LAP2α fused with mCFP also produced FRET signals with mVenus-BAF in the core (data not shown), consistent with the previous finding that LAP2α is colocalized with BAF at the core.
Fig. 1. See next page for legend.
BAF directs nuclear envelope assembly

Table 1. FRET efficiency

|                | Interphase NE | Telophase core region |
|----------------|---------------|-----------------------|
| mCFP-BAF/mVenus-BAF | 12.3±8.7 (n=7) | 26.7±14.2 (n=15)    |
| mCFP-BAF/mVenus-emerin | 15.2±10.6 (n=11) | 14.6±13.2 (n=29)    |
| mCFP-BAF/mVenus-emerin m24 | ND (n=20) | 2.7±1.35 (n=10)    |
| mCFP-BAF/mVenus-Lamin A | 3.1±8.2 (n=10) | 23.0±12.0 (n=31)    |

*FRET efficiency (%)* = [1−(donor intensity before acceptor photobleaching/donor intensity after acceptor photobleaching)] × 100.

probably forms a polymer or a higher-order complex structure with BAF itself in the core region and also directly binds emerin, lamin A and LAP2α, assembling them into the core complex.

**BAF forms an electron-dense complex on the surface of the telophase chromosome mass**

To examine the core structure at high resolution, we used electron microscopy (EM). Because the core structure forms transiently, for only a few minutes, during telophase, we selected specimens by observing living HeLa cells expressing GFP-BAF. During live-cell observation, the cell was fixed when the core structure formed and embedded in epoxy resin to prepare EM specimens (Fig. 4A; see Materials and Methods). Three-dimensional fluorescence microscopy images of the fixed cell were also obtained (Fig. 4B). Thin EM sections were compared with fluorescence microscopy images to identify the cell observed during live-cell observation (Fig. 4C,D) and details of the core structure were investigated at high resolution (Fig. 4E,F). Images shown in a, b, and c in Fig. 4F correspond to the regions indicated in Fig. 4E. This method provides an opportunity to combine the temporal information and molecular selectivity of fluorescence live-cell imaging with the high-resolution imaging of EM; this method is designated live correlative light electron microscopy (live CLEM). Examples fixed at later stages are shown in Figs 5 and 6. The reliability of this method was confirmed in an immunoelectron micrograph for GFP-BAF localization using anti-GFP antibody (supplementary material Fig. S1): GFP-BAF was localized in the core region of telophase cells as seen in the live CLEM imaging technique.

We applied live CLEM to cells fixed at approximately 4 minutes (n=4), 7 minutes (n=17) and 10 minutes (n=3) after the metaphase-anaphase transition, (Figs 4, 5 and 6, respectively). The cell shown in Fig. 4 was fixed at 4 minutes 15 seconds after the metaphase-anaphase transition, before BAF became concentrated at the core. Comparison of EM images with those from fluorescence microscopy shows that...
Fig. 3. FRET analyses of core-localizing NE proteins. (A-H) HeLa cells transiently expressing two fusion proteins, as indicated, were analyzed by the acceptor photobleaching method: FRET signals were detected by an increase of donor fluorescence after photobleaching the acceptor chromophore. (A,C,E,G) Typical examples of images at 480 nm for mCFP and 520 nm for mVenus. The bleached area is indicated by the white square; the inset in the upper right corner is an enlarged image of the bleached area. (B,D,F,H) Fluorescence spectra measured in the bleached area before and after acceptor photobleaching. (I) HeLa cells transiently expressing CFP-BAF and YFP-BAF were analyzed by the ratio-imaging method. Images were obtained every minute. Time 0 represents timing of the metaphase-anaphase transition. Ratio images are represented by the color code shown on the right. Scale bars: 10 μm (A,C,E,G).
BAF was localized to only a limited region of the chromosome mass (superimposed with the green region in panel b of Fig. 4F); this region represents a trailing edge of the separating chromosomes, which might correspond to the telomeres, as suggested by Foisner and colleagues (Dechat et al., 2004). In the central region of the chromosomes where BAF localizes, spindle MTs approached and penetrated into the chromosome mass (superimposed with the orange lines in regions b and c of Fig. 4F). At this time, the NE (superimposed with the red hatches) was not being formed in the central region of the chromosome mass where the mitotic spindle was present; rather, the most peripheral regions (‘non-core’ region in Fig. 1A) of the chromosome mass were being enveloped by the NE (see panel a of Fig. 4F; also see supplementary material Fig. S2A,B). The purple arrowheads indicate vesicular membranes. These vesicular membranes are presumably part of the ER tubules, the precursors of nuclear membranes (Anderson et al., 2007). It should be emphasized that almost no NE was formed in the central region (b and c in Fig. 4F) where spindle MTs are present, whereas the NE was well defined in the peripheral regions where spindle MTs are few or not present at all.

The cell shown in Fig. 5 was fixed at 7 minutes 10 seconds after the metaphase-anaphase transition, when distinct core localization of BAF was observed. Strikingly, an electron-dense area, with a width of 24-64 nm, overlapping with the localization of BAF (superimposed with green) was clearly seen on the surface of the chromosome mass (Fig. 5G). At this time, well-defined NE could be observed in this region of the chromosome mass, but the NE was not yet fully assembled at the sites of the electron-dense areas (panel a in Fig. 5G). Vesicular membranes could be seen near these electron-dense areas (indicated by purple).

Fig. 4. Live-correlated EM analyses. (A) Time-lapse images of living HeLa cells expressing GFP-BAF were obtained every minute. Magenta represents chromosomes (Hoechst 33342) and green represents GFP-BAF. Cells were fixed after live-cell fluorescence imaging, and the same cell was subjected to EM analysis. (B) A series of deconvolved 3D images of the fixed cell. (C) A single section image from the indicated panel in B. (D) An EM image corresponding to the image in C. (E) Magnified view of the region indicated in D. (F) High-magnification EM images of the regions a-c indicated in E. In the lower panels, drawings are superimposed on the EM images indicating GFP-BAF (green), the NE (red), MTs (orange) and vesicles (purple arrowheads). Scale bars: 10 μm (A,C), 2 μm (D) and 200 nm (F).
arrowheads in Fig. 5G); some vesicles with a diameter of approximately 50 nm appeared to attach to the BAF-containing electron dense areas (see panel c in Fig. 5G and panel b in Fig. 6G). In addition, vesicular membranes at the site of the reforming NE at the core region appeared to be attached to MTs (see arrowheads in panel c in Fig. 5G and panel b in Fig. 6G). It could also be seen that some MTs had penetrated into the chromosome mass in the gap between two BAF-containing electron-dense areas (panel b in Fig. 5G), and others attached to the surface of the electron-dense area (panel d in Fig. 5G). At the same time, most...
Fig. 6. Live-correlated EM analyses. (A) Time-lapse images of living HeLa cells expressing GFP-BAF were obtained every minute. Magenta represents chromosomes (Hoechst 33342) and green represents GFP-BAF. Cells were fixed after live-cell fluorescence imaging, and the same cell was subjected to EM analysis. (B) A series of deconvolved 3D images of the fixed cell. (C) A single section image from the indicated panel in B. (D) An EM image corresponding to the image in C. (E,F) Magnified views of the regions indicated in D. (G) High-magnification EM images of the regions a-c indicated in E and F. In the lower panels, drawings are superimposed on the EM images indicating GFP-BAF (green), the NE (red), MTs (orange), and vesicles (purple arrowheads). (H) Schematic diagram of NE reformation during telophase. Upper panel: cartoon representation of NE formation at the core region. BAF (green) forms a stable complex on the surface of the telophase chromosome mass in the presence of MTs. Then, LEM-domain proteins containing NE precursor vesicles attach to the BAF complex, fuse with each other, and finally enclose the chromosomes at the core region as MTs disassemble. Lower panel: EM images of the stages depicted by the cartoon representations. Scale bars: 10 μm (A,C), 2 μm (D), 200 nm (G) and 100 nm (H).
Fig. 7. See next page for legend.
BAF has a direct role on NE formation at the core region, and lamin A and emerin affect the lifetime of the core structure

To determine the contribution of BAF to core formation and subsequent NE formation, we examined the effects of BAF knockdown by RNAi in HeLa cells. BAF was selectively removed in cells treated with BAF siRNA (Fig. 7A,D). In those cells, core localization of emerin and lamin A was effectively inhibited (Fig. 7B); examples of microscopic images are shown in Fig. 7C. In addition, we applied live CLEM analysis to BAF siRNA-treated HeLa cells expressing GFP-BAF, and observed cell structures especially focusing on the electron-dense structure, the NE, chromosomes, the MTs and membranes (Fig. 7E, Figs 8 and 9; also see supplementary material Fig. S4). In BAF siRNA-treated cells (see Fig. 7D), the electron-dense structure at the periphery of the central region of the chromosome mass was lost (n=4; Fig. 7E, compare arrowheads in cells at 7 minutes after the metaphase-anaphase transition with arrows in the control cells), and chromosomes remained condensed (Fig. 8, compare G or M with B), indicating that BAF is required for formation of the core structure and for chromosome decondensation. In addition, the NE at the core region had not formed in BAF siRNA-treated cells even at 12 minutes after the metaphase-anaphase transition (n=10; see Fig. 8J) whereas it was almost fully reassembled in luciferase siRNA-treated control cells (n=4; Fig. 8D). In the BAF-depleted cells it was also frequently observed that relatively long MTs remained in the chromosomes (compare Fig. 8J with 8D; also compare Fig. 9F with 9C). Finally, abnormal inclusion of cytoplasm inside the nucleus was also often seen these cells (Fig. 8P,Q; pale regions in nucleus).

Interestingly, in addition to these phenotypes, BAF depletion caused a significant reduction of tubular membranes near the core region where the spindle MTs persisted, in contrast to an abundance of tubular membranes in the presence of BAF (compare Fig. 9E,F with 9B,C). Instead, in BAF RNAi cells, abnormal piles of three-dimensionally extended ER membranes in the cytoplasm were generated (Fig. 9G,H; also see Fig. 8K,L, compare these with Fig. 8E): in many cases these abnormal membranes seemed to be NE-like membranes assembling away from the chromosomes (Fig. 9G,H). These findings are schematically summarized in Fig. 9I. Taken together, these results indicate that BAF has a direct effect on NE formation at the core region, as suggested previously (Haraguchi et al., 2000), and suggest that the role of BAF in NE formation is to coordinate the recruitment of NE precursor membranes to the chromosome surface.

RNAi knockdown of lamin A did not eliminate core localization of BAF or emerin, but did reduce the frequency of core localization (Fig. 7B). To test whether this reduced frequency reflected a shortened life of the core structure, we determined the duration of the core structure (Fig. 7F) in cells with specific RNAi-mediated knockdown of BAF, lamin A or emerin, as shown in Fig. 7D. Its duration was significantly shortened from 8 minutes 9 seconds (n=10) in control luciferase siRNA cells to 1 minute 42 seconds (n=7) in lamin A siRNA cells, suggesting that lamin A stabilizes the core structure (Fig. 7F). By contrast, in emerin siRNA cells, the core structure remained intact significantly longer (more than 20 minutes) (n=7) compared with control RNAi cells (n=7) (Fig. 7F), often persisting up to early G1 phase (data not shown), suggesting that emerin destabilizes the core structure.

Furthermore, knockdown of these proteins also affected localization to the NE in the subsequent interphase. Knockdown of BAF disrupted NE localization of emerin and lamin A,
knockdown of lamin A disrupted NE localization of BAF and emerin, and knockdown of emerin disrupted NE localization of BAF and lamin A (Fig. 7G). These effects are specific because the same treatment did not affect lamin B localization (Fig. 7G). Taken together, these results suggest that BAF has a direct role on core formation and that lamin A and emerin affect the stability of the core structure.

The spindle MTs mediate BAF assembly to the core region. BAF forms an electron-dense core structure close to the MT-attaching regions (Fig. 5). Therefore, the MTs could be involved in BAF assembly to the core region. To test this idea, we first examined whether BAF colocalizes with the spindle MTs during mitosis. Indirect immunofluorescence staining of cells fixed with trichloroacetic acid (TCA) showed that in mitotic cells BAF

![Image](https://example.com/image1.png)

Fig. 8. Live-correlated EM analyses of HeLa cells after siRNA treatment for BAF. (A) Time-lapse images for chromosomes and GFP-BAF were taken for control cells. Cells were fixed at 12 minutes after the metaphase-anaphase transition. Only images for GFP-BAF are shown. See supplementary material Fig. S4 for more details. (B) An EM image of the same cells shown in A. (C) A fluorescence micrograph of the same cells shown in A: selected focus from a series of 3D images. Magenta represents chromosomes (Hoechst 33342) and green represents GFP-BAF. (D) Magnified view of the region indicated in the larger square in B. Drawings are superimposed on the EM images indicating the NE (red) and MTs (orange). (E) High-magnification EM images of the small box indicated in B. (F) Time-lapse images for chromosomes and GFP-BAF were taken for BAF RNAi cells. Cells were fixed at 12 minutes after the metaphase-anaphase transition. Only images for GFP-BAF are shown. See supplementary material Fig. S4 for more details. (G) EM images of the same cells shown in F. (H) A fluorescence micrograph of the same cells in F: a selected focus from a series of 3D images. Magenta represents chromosomes (Hoechst 33342) and green represents GFP-BAF. (I) Magnified view of the region indicated in the largest square in G. (J) Drawings are superimposed on the EM images of I, indicating the NE (red) and MTs (orange). (K,L) High-magnification EM images of the small boxes indicated in G: K, left box; L, right box. (M) An EM image of another example of BAF siRNA-treated cells. (N) A fluorescence micrograph of the cells shown in M. Magenta represents chromosomes (Hoechst 33342) and green represents GFP-BAF. (O) Enlarged image of the boxed region in N and corresponding to the boxed region in M. (P) Magnified view of the boxed region in M and corresponding to the image shown in O. (Q) Drawings are superimposed on the EM images of P, indicating the NE (red) and chromosomes (magenta). Scale bars: 10 μm (A,F), 2 μm (B,C,G,H,M,N), 500 nm (D,J,P,Q) and 200 nm (L).
localizes to the spindle MTs from metaphase to telophase (Fig. 10A). Since this result shows colocalization of BAF with the spindle MTs for the first time, we used BAF knockdown by BAF RNAi to confirm our initial observation; BAF RNAi treatment effectively removed the signal, indicating that BAF, at least in part, colocalizes with the spindle MTs (supplementary material Fig. S5). To further test a direct role of the spindle MTs in core formation, we examined the effect of nocodazole, a MT-depolymerizing reagent, on BAF assembly to the core region. In cells treated with nocodazole during anaphase, the spindle MTs disappeared and BAF core assembly was lost (Fig. 10B), indicating that the spindle MTs are required for BAF assembly to the core. Taken together, these results suggest that BAF is associated with the spindle MTs during metaphase through anaphase and assemblies to the core region in early telophase, in a spindle-MT-dependent manner, where it forms a stable complex with other core-localizing proteins such as lamin A and emerin. Based on these findings, we propose a model, schematically illustrated in Fig. 10C, in which spindle MT function organizes BAF spatially, thereby engendering BAF-directed NE assembly.

Discussion
In this report, we have demonstrated that the BAF-dependent core structure is an immobile complex that appears as an electron-dense structure directly on chromosomes with a width of 24-60 nm. This study shows for the first time that the spindle MTs are involved in NE reformation at the end of mitosis: spindle MTs mediate BAF assembly to the core region where BAF recruits selective NE components such as lamin A and emerin. The core structure is important in establishing a normal interphase NE and, consequently, normal nuclear function.

Live CLEM observation revealed that the timing of NE reformation is different in the core region from that in the non-core region: NE reformation is significantly later in the core region (compare Figs 4-6 with supplementary material Fig. S2). This suggests that there are at least two different mechanisms for NE assembly; one that acts at the core region, and a second that acts at the non-core region. RanGTP would appear to be involved in NE reformation at the non-core region because it is known to be involved in the assembly of the NPCs in in vitro Xenopus oocyte extracts.

Fig. 9. Live-correlated EM analyses of HeLa cells expressing GFP-BAF after siRNA treatment (D-H) or luciferase siRNA treatment as a control (A-C). During time-lapse imaging of living HeLa cells expressing GFP-BAF, cells were fixed 7 minutes after the metaphase-anaphase transition and subjected to EM analysis. A magnified view of the region indicated in A is shown in B and C. Magnified views of the regions indicated in D are shown in E and F (left square region), and G and H (right square region). In C, F and H, drawings are superimposed on the EM images indicating GFP-BAF (green), the NE (red), and MTs (orange). Scale bars: 2 μm (A,D), 500 nm (B,C,E-H). (I) Model of NE formation in the control siRNA-treated (left) and BAF siRNA-treated cell (right). For details, see text.
findings strongly support the idea that BAF acts to direct core NE targeting to the core region requires spindle MTs (Fig. 10B). These observations, we report that BAF associates with the spindle MTs, and that its association with the spindle MTs.

As BAF is a key molecule for core formation, BAF targeting to the core region must be an important step for core formation. Recently, Gorjánácz and colleagues have reported that BAF-1 (C. elegans BAF homolog) is localized in a chromosomal region similar to the ‘core’ during NE formation in dividing cells of early C. elegans embryos, and its ‘core’-like localization is abolished by siRNA depletion of VRK-1 (C. elegans vaccinia-related kinase homolog) (Gorjánácz et al., 2007). Their results suggest that phosphorylation of BAF is essential for its ‘core’-like localization in C. elegans embryos. Human BAF can be phosphorylated in vitro on its N-terminal conserved amino acid residues, Ser4 (Bengtsson and Wilson, 2006) or all three amino acid residues Thr2-Thr3-Ser4, by vaccinia-related kinases (Nichols et al., 2006). Paradoxically, however, initial studies have indicated that phosphorylation of human BAF abolishes its interaction with DNA in vitro (Nichols et al., 2006), reduces its binding to the LEM proteins in vitro (Nichols et al., 2006), and disrupts emerin localization to the NE in vivo (Bengtsson and Wilson, 2006), suggesting that the N-terminal phosphorylated form of human BAF cannot attach to the chromosomal region at the ‘core’. One possibility to reconcile these results is that BAF phosphorylated by VRK1 binds spindle MTs, and then is able to localize to the core region once it is dephosphorylated at the end of mitosis. This hypothesis is supported by the following findings: (1) BAF associates with the spindle MTs (Fig. 10A); (2) BAF core accumulation is abolished by destruction of the spindle MTs (Fig. 10B); and (3) phosphorylated BAF is localized on the spindle MTs but not in the core region (T.H., unpublished result). However, it remains to be elucidated whether phosphorylation by Vrk1 is required for association with the spindle MTs.

Cell-cycle dependent phosphorylation of emerin also regulates the interaction of emerin with BAF (Hirano et al., 2005; Ellis et al., 1998), suggesting that modification of emerin or other core-localizing proteins may also contribute to the core formation and structural features of the ‘core’. Since mitotic progression is regulated by the spatial and temporal coordination of several cell-cycle-regulating kinases and phosphatases, the combination of several protein modifications may cooperatively enforce core formation in telophase.

Cooperative actions of BAF and core-localizing proteins in NE organization are supported by several observations. BAF, lamin A and emerin form a three-way complex in vitro (Holaska et al., 2003) and in vivo in C. elegans (Liu et al., 2003; Margalit et al., 2005; Gorjánácz et al., 2007). Retention of lamin A and emerin in the reforming NE requires their initial localization at the core region, and this localization is BAF dependent (see Fig. 7G). Core localization is also required for BAF to establish its own proper nuclear localization (Zheng et al., 2000; Margalit et al., 2005; Gorjánácz et al., 2007). Furthermore, the lifetime of the core structure seems to depend on specific core-localizing proteins such as emerin, lamin A and LAP2α. Loss of emerin by RNAi prolonged core lifetime (see Fig. 7F). A persistent core structure is also
observed in HeLaS3 cells expressing high levels of lamin A, and this persistent core structure is enriched in emerin and lamin A (Maeshima et al., 2006). We also observed a prolonged core lifetime in cells overexpressing lamin A or LAP2α (data not shown). By contrast, loss of lamin A by RNAi significantly reduced the lifetime of the core structure (see Fig. 7F). These results suggest that emerin destabilizes the core, whereas lamin A and LAP2α stabilize the core structure. The balance between their competing activities may be important for regulating the formation of the transient core structure, the formation of the mature NE and the eventual formation of a functional nucleus.

In summary, we conclude that BAF assembles on the surface of the telophase chromosome mass in regions where the chromosomes are interacting with spindle MTs, and that this assembly is MT dependent and requires coordinated BAF phosphorylation followed by BAF dephosphorylation. Chromosome-associated BAF makes a higher-order complex with itself, lamin A and emerin, resulting in formation of an electron-dense structure at the core which is required for proper reformation of the NE in this region and for the re-establishment of a functional nucleus. Furthermore, BAF-dependent NE reformation at the core, which involves LEM-domain proteins containing NE precursor vesicles attaching to the BAF complex and then fusing with each other, is mechanistically distinct from NE reformation at the non-core region of the chromosome mass. Understanding the dynamics and the structural details of NE reformation at the end of mitosis will provide new insights into the development of lamin-A-dependent disease and other NE-associated human diseases.

Materials and Methods

Cells and reagents

HeLa cells were obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in DME medium containing 10% calf serum. Double-stranded siRNA for BAF, lamin A and emerin were purchased from Qiagen (Hilden, Germany). Mouse monoclonal anti-lamin A/C antibodies TIM29 (IgM) were generated as described previously (Haraguchi et al., 2001). Mouse monoclonal anti-lamin A/C antibodies MMS-107P (IgM) were purchased from Covance (Berkeley, CA). Mouse monoclonal anti-lamin B (101-B7) antibodies were obtained from MatriTech (Cambridge, MA). Rabbit polyclonal anti-emerin antibodies were a gift of the Arakawa lab (Tokyo, Japan). Anti-BAF antibodies (PU38143) were generated against recombinant BAF in a rabbit and affinity purified. Plasmids encoding mCFP and mVenus (monomeric versions of ECFP and Venus), which were made by substitution of Ala for Lys (Zacharias et al., 2002), were gifts of Nagai, Sawano and Miyawaki (RIKEN, Wako, Japan). LAP2α-YFP and GFP-lamin B1 were gifts of R. Foisner (Medical University Vienna) and R. Goldman (Northwestern University), respectively.

Plasmid construction

Methods for constructing DNA plasmids encoding LBR-GFP, GFP-emerin, GFP-BAF, GFP-LAP2β and GFP-MAN1 were described previously (Haraguchi et al., 2000; Haraguchi et al., 2001, Shimi et al., 2004). To construct GFP-lamin A, the coding region was PCR-amplified from the RT-PCR product of lamin A using primers lam1 and lam2 and inserted into the pEGFP-C1 vector (Clontech Laboratories, Palo Alto, CA). To construct mCFP and mVenus fusion proteins, the coding region of mCFP or mVenus was PCR-amplified from ECFP-A206K/prSETB or Venus-A206K/prSETB, respectively, with primers cfp-1 and cfp-2 and inserted into the pEGFP-C1 vector digested with Nhel and BglII restriction enzymes to replace ECFP. The resulting mCFP and mVenus vectors were used for construction of mCFP-BAF, mCFP-lamin A, mCFP-emerin, mCFP-emerin-m24 and mVenus-BAF. To construct mCFP-BAF or mVenus-BAF, the coding region of BAF was PCR-amplified from pET15b-BAF (Lee and Craigie, 1998) using primers baf-1 and baf-2 and inserted into the above described mCFP or mVenus vectors, respectively. To construct mVenus-emerin or mVenus-emerin-24m, the coding region of emerin was PCR-amplified from a GFP-emerin construct (Haraguchi et al., 2000) using primers eme-1 and eme-2 and inserted into the mVenus vector. To construct mVenus-lamin A, the coding region of lamin A was PCR-amplified from the pEGFP-lamin A construct using primers lam3 and lam4 and inserted into the mVenus vector. To construct mVenus-lamin A, the coding region of lamin A was PCR-amplified from the pEGFP-lamin A construct using primers lam3 and lam4 and inserted into the mVenus vector. To construct mVenus-lamin A, the coding region of lamin A was PCR-amplified from the pEGFP-lamin A construct using primers lam3 and lam4 and inserted into the mVenus vector. To construct mVenus-lamin A, the coding region of lamin A was PCR-amplified from the pEGFP-lamin A construct using primers lam3 and lam4 and inserted into the mVenus vector.

Fluorescence microscopy

Cells were grown in a glass-bottom culture dish (MatTech, Ashland, MA). GFP fusion plasmids (0.1-0.2 μg) were transfected into cells with LipofectaminePlus (Gibco BRL) according to the manufacturer’s methods except that the incubation time with DNA was reduced to 1.5 hours.

Time-lapse, multicolor images in living cells, as well as indirect immunofluorescence images in fixed cells, were obtained using the DeltaVision microscope system based on an Olympus wide-field fluorescence microscope IX70 (Applied Precision, Seattle, WA). For temperature control during live observation, the incubator (Wener) was kept at 1 hour and 40 min on a temperature-controlled stage, as described previously (Haraguchi et al., 1999). A series of three-dimensional image data were obtained, and deconvolved with the Softworx software equipped with the DeltaVision as described previously (Haraguchi et al., 2001; Haraguchi et al., 2004).

FRAP and FRET analyses

FRAP and FRET studies were done using a laser-scanning confocal microscope, Zeiss LSM510 META (Carl Zeiss, Jena) equipped with a Kr laser (Coherent, Santa Clara, CA) (Haraguchi et al., 2002; Hiraoka et al., 2002). FRAP experiments were carried out as described previously (Shimi et al., 2004). Briefly, HeLa cells stably expressing GFP fusion proteins were imaged using a water-immersion objective lens (C-Apochromat 40×/NA1.2) on a Zeiss LSM 510 META. Photo bleaching was performed with full power of 488 nm light from the argon laser 10 iterations for a defined region of each cell. Image data were collected through a pinhole of 2.37 Airy units (corresponding to an optical section 2 μm thick) with 488 nm argon laser at 0.5% full power.

For FRET experiments, living HeLa cells transiently expressing pairs of NE proteins fused with mCFP and mVenus were imaged using a water-immersion objective lens (C-Apochromat 40×/NA1.2) on a Zeiss LSM 510 META. FRET was detected by acceptor photobleaching (Wouters et al., 2001). mVenus was photobleached by 514 nm light from the argon laser, with full power, 50 iterations for a defined area (2.1 μm square) in each cell. After photobleaching, time-lapse images were collected on the META detector from 470 nm to 545 nm through a pinhole of 2.18 Airy units (corresponding to an optical section 1.8 μm thick) by exciting with 413 nm light from the Kr laser at 0.3-0.9% full power. FRET efficiency was determined for fluorescence intensity of mCFP and mVenus after mCFP and mVenus after linear unmixing as described previously (Shimi et al., 2004). For time-lapse FRET ratio imaging, a fluorescence microscope IX70 (Olympus) equipped with a Nipkow disc confocal unit, CSU21 (Yokogawa, Japan) was used. Cells were illuminated with a 430 nm diode laser to excite CFP, and image data for CFP and YFP were simultaneously obtained on a CoolSnapHQ CCD camera (Photometrics, Reper, Tucson, Texas) through a dual viewer (Optical Insights), which separates emission light for wavelengths of 480 nm (corresponding to CFP) and 535 nm (corresponding to YFP). Ratio images between CFP and YFP were obtained by MetaMorph software (Universal Imaging Corporation, Downingtown, PA).

Correlative light electron microscope analysis after live observation (live CLEM)

Cells stably expressing GFP-BAF were cultured in a special glass-bottomed dish with an addressing grid (grid size, 175 μm) on the coverslip (Iwaki, Japan). After staining with Hoechst 33342, live cells were monitored on DeltaVision; at the desired time point after the metaphase-anaphase transition, cells were fixed with glutaraldehyde at a final concentration of 2.5% for 1 hour. Three-dimensional images (50-60 focal planes at 0.2 μm intervals) were obtained using an Olympus oil immersion objective lens (PlanApo ×60/1.4) and computationally processed by three-dimensional deconvolution (Agard et al., 1989). Electron microscope (EM) observation of the same cell was carried out as follows. Cells were post-fixed with 1% OsO4 (Nissin EM, Tokyo, Japan) in phosphate buffer, pH 7.4, for 1 hour, washed briefly with distilled water, and sequentially dehydrated with 50% and 70% ethanol. After staining with 2% uranyl acetate in 70% ethanol for 1 hour, cells were again sequentially dehydrated with 90% and 100% ethanol, and then embedded in epoxy resin by incubating with 50% (v/v) Epon812 (TAAB, Berkshire, UK) in ethanol for 30 minutes and 100% Epon812 for 1 hour. The epoxy block containing the same cell observed by a fluorescence microscope was trimmed according to the grid reference on the coverslip, and sliced to ultra-thin sections with a thickness of 80 nm using a microtome (Leica microsystems, Germany). Thin sections were stained with 2% uranyl acetate (Merck) for 1 hour and 40 min on a temperature-controlled stage for lead citrate (Sigma) for 1 minute. Image data were collected using an electron microscope Hitachi H7600 (80 kV, Hitachi, Japan).

Knockdown by RNAi

Two short micro oligonucleotides of oligomeric double-stranded RNA for BAF, lamin A or emerin were transfected into HeLa cells cultured in a 35 mm culture dish using Oligofectamine (Invitrogen, Carlsbad, CA), Lipofectamine PLUS (Invitrogen), or RNAiFect (Qiagen) transfection reagents according to the manufacturer’s instructions. The cells were incubated for 3 days in DMEM culture medium containing 10% calf serum.

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